Ⅷ. 研 究 事 業 報 告

厚生科学研究費補助金難治性疾患克服研究事業
「炎症性腸疾患の画期的治療法に関する臨床研究」
平成16年度第1回総会プログラム

（敬称略）

開会（13：00）

Ⅰ．主任研究者挨拶・研究の進め方　　班長：渡辺　守

Ⅱ．研究報告

（1）上皮細胞の再生・修復のための分子療法の確立（13：10〜14：05）

・遺伝子組み換え型ヒトHGFを用いたTNBS大腸炎に対する粘膜修復治療

（分担研究者：坪内博仁）

坪内博仁、○沼田政嗣、井戸章雄、安部弘生、宇都浩文

（宮崎大学第2内科、京都大学探索医療センター）

・炎症性腸疾患に対するHGF遺伝子治療の検討　（分担研究者：鈴木健司）

○鈴木健司、河内裕介、朝倉　均、青柳　豊　（新潟大学大学院医歯学系消化器内科学分野）

・炎症性腸疾患にみられる腸上皮の再生・分化異常と腸上皮幹細胞の検討

（分担研究者：高後　裕）

○綾部時芳、蘆田知史、高後　裕　（旭川医科大学第3内科）

・腸管上皮細胞の分化制御による新規治療法開発への試み　（分担研究者：渡辺　守）

○岡本隆一、松本智子、川村央信、土屋輝一郎、中村哲也、金井隆典、渡辺　守

（東京医科歯科大学大学院消化器病態学）

（2）腸管特異的免疫調節を応用した治療法の開発（14：05〜14：55）

・MIFの制御による炎症性腸疾患の新しい治療法の開発　（分担研究者：浅香正博）

○大川原辰也、武田宏司、浅香正博　（北海道大学大学院消化器内科学分野）

・マウスDSS腸炎発症における$\gamma\delta$-IELの役割　（分担研究者：石川博通）

○石川博通　（慶應義塾大学微生物学・免疫学）

・リンパ球ホーミング作動薬FTY720によるクローン病緩解維持療法に対する臨床応用

○伊藤壽記、水島恒和、玉川浩司、甲斐康之、根津理一郎　（大阪大学大学院臓器制御外科）

・自然免疫系からみた炎症性腸疾患の病態解析　（分担研究者：竹田　潔）

○竹田　潔　（九州大学生体防御医学研究所発生工学分野）（※紙上発表）

（3）選択的細胞除去・移入療法の開発（14：55～15：30）

・炎症性腸疾患における制御性Ｔ細胞：疾患におけるその割合の変動とヒト末梢血よりの分離法、機能に関する検討　（分担研究者：中村和彦）

○中村和彦、高橋　誠、水谷孝弘、本田邦臣、松井謙明、吉永繁高、秋穂裕唯、名和田　新

（九州大学大学院・病態制御内科学）

・細胞治療としての炎症性腸疾患における免疫抑制性Ｔ細胞移入療法実現に向かって

（分担研究者：渡辺　守）

○金井隆典、河村貴広、蒔田　新、浦牛原幸治、戸塚輝治、渡辺　守

（東京医科歯科大学大学院消化器病態学）

（4）分子デリバリーシステムを用いた治療法確立（15：30～15：45）

・潰瘍性大腸炎患者におけるデキサメサゾン含有ポリ乳酸マイクロカプセルの有効性に関する研究　（分担研究者：岡崎和一）

○岡崎和一、松下光伸、川股聖二、渡辺敏彦、斎藤一文字、西尾彰功、仲瀬裕志、千葉　勉、乾　賢一、田畑泰彦

（関西医科大学第３内科、関西医科大学薬剤部、京都大学消化器内科、京都大学薬剤部、京都大学再生医学研究所）

（5）新しいコンセプトによる治療法開発（15：45～16：20）

・好中球エラスターゼ阻害剤によるマウスDSS腸炎治療効果の検討

（分担研究者：日比紀文）

○諸星雄一、松岡克善、久松理一、岡本　晋、高石官均、井上　詠、日比紀文

（慶應義塾大学消化器内科、慶應義塾大学病院包括先進医療センター）

・炎症性腸疾患における薬剤抵抗性の克服

○有村佳昭，後藤　啓，小林歓和，岡原　聡、中原生哉、今井浩三　（札幌医科大学第１内科）

事務局連絡

閉会の挨拶

平成16年度第1回総会出席者名簿

平成16年7月30日（金）
参加者64名（敬称略）

班　　長　渡辺　守（東京医科歯科大学消化器内科）

分担研究者　浅香正博（北海道大学医学部第3内科）
坪内博仁（宮崎大学医学部第2内科）
高後　裕（旭川医科大学第3内科）
岡崎和一（関西医科大学第3内科）
石川博通（慶應義塾大学微生物学・免疫学）
中村和彦（九州大学大学院病態制御内科）
鈴木健司（新潟大学消化器内科）

参加協力者　井上　詠、松岡克善、諸星雄一（慶應義塾大学消化器内科）
武田宏司（北海道大学医学部第3内科）
井戸章雄、沼田政嗣（京都大学医学部附属病院探索医療センター）
安倍弘生、宇都浩文（宮崎大学医学部第2内科）
綾部時芳（旭川医科大学第3内科）
千葉　勉、西尾彰功（京都大学消化器内科）
松下光伸（関西医科大学第3内科）
高橋　誠（九州大学大学院病態制御内科）
伊藤壽記、水島恒和、玉川浩司、根津理一郎、松田　宙（大阪大学大学院臓器制御外科）
有村佳昭（札幌医科大学第1内科）
岡本哲郎（札幌医科大学第4内科）
矢花　崇、本谷　聡（札幌厚生病院消化器科）
渡邉聡明（東京大学腫瘍外科）
亀岡信悟（東京女子医科大学外科）
飯塚文瑛（東京女子医科大学消化器内科）
三浦総一郎（防衛医科大学校第2内科）
大塚和朗（昭和大学横浜市北部病院消化器センター）
五十嵐正広（北里大学内科）
杉村一仁（新潟大学医学部第3内科）
中野　浩（藤田保健衛生大学内科）
伊藤裕章（大阪大学消化器内科）
渡辺憲治（大阪市立大学医学部第3内科）
髙木智久、内藤裕二（京都府立医科大学消化器内科）
樋田信幸、福永　健（兵庫医科大学下部消化管科）
松本啓志（川崎医科大学内科）
伊藤浩史（宮崎大学第2病理）
光山慶一（久留米大学第2内科）
牧山和也（長崎大学光学医療診療部）
柴田　博、角　隆行（旭メディカル）
岡本正人（田辺製薬）
菅原慎一郎、藤井克典、丸田展久（日清キョーリン製薬）
細井英治、槌屋紘典（日本抗体研究所）
岡本泰子（藤澤薬品）
金井隆典、岡本隆一（東京医科歯科大学消化器内科）

事　務　局　山崎元美、谷本佳奈美、児玉　瞳、藤井　玲、伊藤裕子（東京医科歯科大学消化器内科）

厚生科学研究費補助金難治性疾患克服研究事業
「炎症性腸疾患の画期的治療法に関する臨床研究」
平成16年度第2回総会プログラム

（敬称略）

開会（9：00）

Ⅰ．主任研究者挨拶・研究の進め方　　　班長：渡辺　守

Ⅱ．研究報告

◎ 上皮細胞の再生・修復のための分子療法の確立（9：10～10：05）

1）組み換え型ヒトHGFの臨床応用における問題点　（分担研究者：坪内博仁）
坪内博仁、〇井戸章雄、沼田政嗣、宇都浩文（宮崎大第二内科）

2）炎症性腸疾患に対するHGF遺伝子治療法開発の検討　（分担研究者：鈴木健司）
〇鈴木健司、河内裕介、朝倉　均、青柳　豊（新潟大学大学院医歯学総合研究科消化器内科学分野）

3）腸上皮の自然免疫機構を標的とする炎症性腸疾患治療法開発　（分担研究者：高後　裕）
〇綾部時芳、蘆田知史、伊藤貴博、田邊裕貴、前本篤男、高後　裕（旭川医科大学第三内科）

4）腸管上皮の分化制御による上皮再生治療の可能性　（分担研究者：渡辺　守）
〇岡本隆一、松本智子、川村央信、大島　茂、土屋輝一郎、中村哲也、金井隆典、渡辺　守
（東京医科歯科大学大学院消化器病態学）

◎ 腸管特異的免疫調節を応用した治療法の開発（10：05～10：45）

5）MIF（macrophage migration inhibitory factor）の制御による炎症性腸疾患の新しい治療法の開発　（分担研究者：浅香正博）
〇武田宏司、大川原辰也、浅香正博　（北海道大学大学院消化器内科学）

6）TCRα欠損マウスIBD発症におけるγδT細胞の役割　（分担研究者：石川博通）
〇石川博通（慶應義塾大学微生物学・免疫学）

7）自然免疫系による慢性炎症性腸疾患の制御機構　（分担研究者：竹田　潔）
〇竹田　潔（九州大学生体防御医学研究所発生工学分野）

◎ 選択的細胞除去・移入療法の開発（10：45〜11：15）

8) 制御性Ｔ細胞保存を目的とした選択的血球成分除去療法の開発：これまでの成果と今後の展望に関して　（分担研究者：中村和彦）

○中村和彦、高橋　誠、本田邦臣、水谷孝弘、吉永繁高、松井謙明、秋穂裕唯、名和田　新

（九州大学大学院医学研究院病態制御内科）

9) 細胞治療としての炎症性腸疾患における免疫抑制性Ｔ細胞移入療法 －第２報－

（分担研究者：渡辺　守）

○金井隆典、河村貴広、蒔田　新、浦牛原幸治、戸塚輝治、渡辺　守

（東京医科歯科大学大学院消化器病態学）

◎ 分子デリバリーシステムを用いた治療法確立（11：15〜11：30）

10) 潰瘍性大腸炎患者におけるデキサメサゾン含有ポリ乳酸マイクロカプセルの有効性に関する研究　（分担研究者：岡崎和一）

○岡崎和一[1]、松下光伸[1]、川股聖二[1]、渡辺敏彦[1]、斎藤一文字[2]、西尾彰功[3]、仲瀬裕志[3]、千葉　勉[3]、田畑泰彦[4]（[1] 関西医科大学内科学第三講座、[2] 関西医科大学薬剤部、[3] 京都大学消化器内科、[4] 京都大学再生医科学研究所）

◎ 新しいコンセプトによる治療法開発（11：30〜11：45）

11) rebamipide 注腸による潰瘍性大腸炎に対する新規治療法　（分担研究者：日比紀文）

○井上　詠、泉谷幹子、諸星雄一、桜庭　篤、松岡克善、久松理一、岡本　晋、緒方晴彦、岩男　泰、日比紀文（慶應義塾大学消化器内科、慶應義塾大学病院包括先進医療センター）

事務局連絡

閉会の挨拶

平成16年度第2回総会出席者名簿

平成17年1月26日（水）
参加者64名（敬称略）

班　　長	渡辺　守（東京医科歯科大学消化器内科）
分担研究者	日比紀文（慶應義塾大学消化器内科）
	坪内博仁（宮崎大学医学部第2内科）
	高後　裕（旭川医科大学第3内科）
	岡崎和一（関西医科大学第3内科）
	石川博通（慶應義塾大学微生物学・免疫学）
	中村和彦（九州大学大学院病態制御内科）
	鈴木健司（新潟大学消化器内科）
	竹田　潔（九州大学生体防御医学研究所発生工学分野）
参加協力者	井上　詠、高石官均（慶應義塾大学消化器内科）
	武田宏司（北海道大学医学部第3内科）
	井戸章雄（京都大学医学部附属病院探索医療センター）
	沼田政嗣（宮崎大学医学部第2内科）
	綾部時芳（旭川医科大学第3内科）
	松下光伸（関西医科大学第3内科）
	河内裕介（新潟大学消化器内科）
	岡本哲郎（札幌医科大学第4内科）
	鮫島伸一（群馬県立がんセンター外科）
	上野文昭（大船中央病院内科）
	鈴木康夫（東邦大学佐倉病院内科）
	土肥多恵子（国立国際医療センター研究所）
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	味村俊樹（帝京大学医学部外科）
	飯塚文瑛（東京女子医科大学消化器内科）
	大塚和朗（昭和大学横浜市北部病院消化器センター）
	高濱和也、長坂光夫（藤田保健衛生大学消化器内科）
	伊藤裕章（大阪大学消化器内科）
	樋田信幸、高川哲也（兵庫医科大学下部消化管科）
	清水香代子、垂水研一、古賀英樹（川崎医科大学消化器内科）
	光山慶一（久留米大学第2内科）
	牧山和也（長崎大学光学医療診療部）
	栗山敏治、柴田　博、金川章孝、有富正治（旭化成メディカル）
	神田徳雄、佐藤信一、チュア エバン、石田美紀、植村真理、楠　敦、分部浩和、上山直樹（大塚製薬）
	岡本正人（田辺製薬）
	菅原慎一郎、藤井克典、丸田展久（日清キョーリン製薬）
	茅野司郎（日本抗体研究所）
	岡安源浩、桜井伸也（UCBジャパン）
	金井隆典、戸塚輝治、岡本隆一、久保田大輔、蒔田　新（東京医科歯科大学消化器内科）
事 務 局	山崎元美、児玉　瞳、藤井　玲、伊藤裕子（東京医科歯科大学消化器内科）

Ⅸ.　研究成果の刊行物・別刷

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Review

Molecular and clinical basis for the regeneration of human gastrointestinal epithelia

RYUICHI OKAMOTO and MAMORU WATANABE

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

In the gastrointestinal tract, rapid renewal of the epithelium continues throughout life. Therefore, it is believed that the gastrointestinal epithelium has a prominent capacity for regeneration when tissue damage occurs. However, we face some clinical conditions in which regeneration of the gastrointestinal epithelia is severely disturbed. One example is the refractory ulcers seen in the intestine of inflammatory bowel disease patients, and a novel therapy to regenerate damaged intestinal epithelia is earnestly desired in those conditions. Little is known about the maintenance and regeneration of the intestinal epithelia, and a molecular or clinical basis for regenerative medicine is totally lacking at the moment. In this review, we discuss recent findings of the molecules regulating the proliferation and differentiation of epithelial cells. Further study of these molecules may lead to the identification and purification of intestinal stem cells that may be used as a source for transplantation in diseased patients. Endogenous stem cells also could be manipulated to correct dysregulated or prolonged regeneration in diseased patients. Alternatively, we will raise bone marrow cells as another novel source for regenerating the intestinal epithelia. Bone marrow-derived cells are the only cells of extragastrointestinal origin that are shown to contribute to the regeneration of the gastrointestinal epithelia. In bone marrow transplant recipients, donor-derived epithelial cells substantially repopulated the gastrointestinal tract during epithelial regeneration after graft-versus-host disease or ulcer formation. Utilization of these cells may also lead to a novel therapy to regenerate the damaged gastrointestinal epithelia, whether by bone marrow transplantation or by the administration of humoral factors.

Key words: intestinal epithelial cells, regenerative medicine, stem cell, bone marrow cells, inflammatory bowel disease

Introduction

The intestinal epithelium has an outstanding feature compared to other tissues, in that it continues rapid regeneration of the entire tissue throughout life. The renewal is repeated in an extremely short term, for example, within 2–3 days in the small intestine of mice.[1,2] This rapid "build and scrap" is maintained by the proliferating unit, which is composed of a single crypt and the adjacent villi (Fig. 1). Within the villi, there exists a progenitor cell called the intestinal stem cell, which could give rise to every kind of epithelial-lineage cells.[3-5] The gastrointestinal epithelia consists of four lineages of cells; enterocytes, goblet cells, enteroendocrine cells, and Paneth cells.[1] All these mature cells arise from the intestinal stem cell, by migrating upward along the crypt-villi axis towards the tip of the villi, gradually differentiating as they come close to the tip.[2]

However, in some diseases, this rapid regeneration is disturbed, resulting in refractory ulcers in the gastrointestinal tract. In severe radiation injury, the failure to regenerate the gastrointestinal epithelia is critical, and is one of the causes of death.[6] Inflammatory bowel disease, such as ulcerative colitis or Crohn's disease, is a much more common cause of severe epithelial damage in the gastrointestinal tract.[7] In these diseases, refractory ulcers are sometimes fatal, and require total colectomy, or repeated partial resection of the small intestine.[8] Therefore, manipulating epithelial regeneration may provide an alternative treatment that could avoid surgical treatment in these patients.

Bone marrow transplantation for hematological diseases such as refractory anemia or aplastic anemia is a

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Reprint requests to: M. Watanabe

good example of regenerative medicine in clinical practice.[9] This is based on abundant knowledge about the identification, function, and the differentiation of hematopoietic stem cells and also about the regulating molecules leading to mature hematopoietic cells.[10] Compared to this knowledge, intestinal stem cells are yet to be identified, and little is known about how the four different lineages of mature cells arise from a single intestinal stem cell. Thus, much is required to be studied for the establishment of regenerative medicine in the intestinal epithelia.

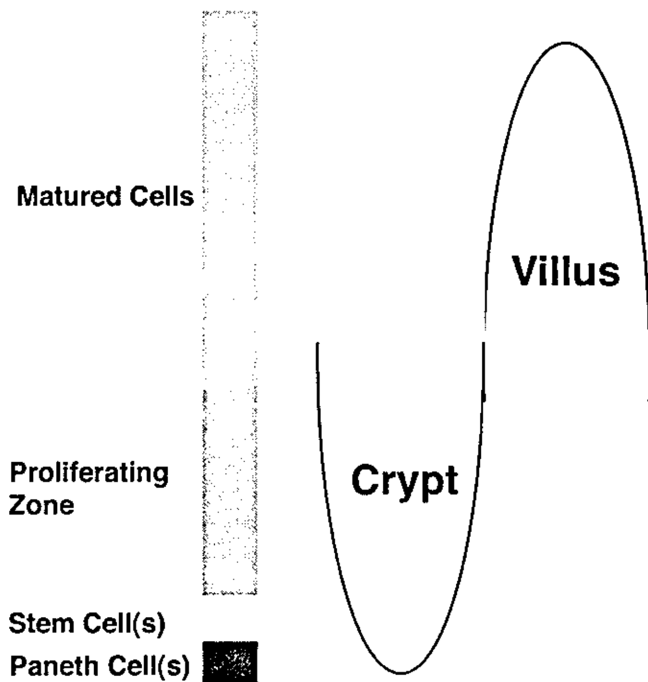
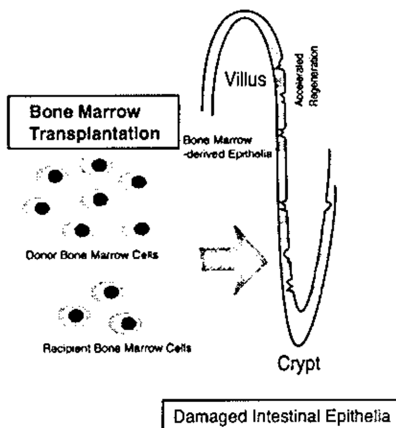


Fig. 1. Unit for the maintenance of the intestinal epithelia (small intestine). A crypt containing stem cells and proliferating progenitor cells provides matured cells to the adjacent villi

In the following sections, we would like to discuss how far we have reached in understanding the maintenance and regeneration of the intestinal epithelia in terms of molecular regulation, and also to consider how we could establish regenerative medicine in the intestinal epithelia from the experimental and clinical evidence we have at the moment.

Identification of intestinal stem cells

Many studies using mouse models have suggested that there exists an intestinal stem cell in the crypt base of the intestinal epithelia,[3-5,11] which could self renew, and, at the same time, give rise to the four main lineages of the intestinal epithelial cells. This is believed to be the same in the human gastrointestinal tract, but has yet to be proved. So far, the predicted intestinal stem cell has not been identified, nor has it been isolated and cultured in vitro, due to the lack of specific molecule markers and difficulty in maintaining longterm cultures of primary cells. Recently, several molecules have been reported to be expressed specifically in the predicted stem cell region within the crypt,[12-14] Beta-catenin is a ubiquitously expressed molecule within the intestinal epithelia, but beta-catenin located within the nucleus is reported to be restricted to the crypt base region.[12] This suggests that the intestinal stem cell requires constitutive Wnt signaling, for some reason, and this is also supported by the finding that Tcf-4 knockout mice fail to develop an intestinal stem cell region.[15] Musashi-1 is an RNA binding protein reported to be enriched in neural stem cells.[16] The function of this molecule is reported to have a close relation to stem cell properties, especially in terms of self renewal.[17] Recently, two reports have revealed that this molecule is expressed predominantly in the stem cell region of the mouse intestinal crypt.[13,14] One of the molecular functions of Musashi-1 as an RNA-binding



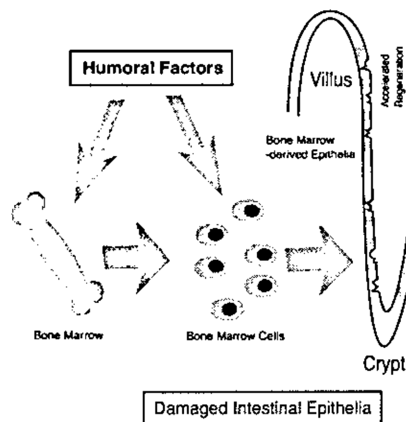


Fig. 2A,B. Hypothetical strategies for using bone marrow cells for the regeneration of the intestinal epithelia. **A** With bone marrow transplantation, adding bone marrow cells that could contribute to the regeneration of the intestinal epithelia might improve epithelial regeneration. **B** Introducing more cells from the patient's bone marrow to damaged epithelia by the infusion of humoral factors might also improve the epithelial regeneration

protein is reported to be that it modulates Notch signaling by repressing the expression of a Notch signal repressor molecule, m-NUMB, in a post-transcriptional manner.[18] Kayahara et al.[14] precisely examined this in the mouse small intestine, and reported that Hes-1, a major transcription factor downstream of Notch signaling, was also expressed in the intestinal crypt region. This suggests that Notch signaling, as well as Wnt signaling, functions in the intestinal crypt of mice, and that this may be regulated or modified by Musashi-1. These molecules might be used as stem cell markers for identifying and isolating intestinal stem or progenitor cells. If a considerable number of intestinal stem cells could be purified, with preserved function, this could be used to reconstitute severely damaged intestinal epithelia, by transplantation into diseased patients. However, whether these molecules directly regulate stem cell function must be proved, and whether the expression and function of these molecules are conserved but altered in the diseased human intestine must be determined. The difficulty in maintaining longterm culture of purified, isolated primary intestinal epithelial cells is another problem that must be solved to make use of human-derived intestinal stem cells.

Role of Wnt signaling in the intestinal epithelia

As mentioned in the section above, recent studies have revealed that Wnt signaling has distinct functions within the intestinal crypt, especially in the stem cell region.[15] Wnt signaling has long been studied in the development of colon cancer, as it is well known that mutation of the gene *apc*, a molecule downstream of Wnt signaling, has a critical role in de-novo tumorigenesis.[19,20] The physiological role of Wnt signaling in the maintenance of the intestinal epithelia remained unclear, until various series of studies in mice were reported.[12,15,21,22] Now, Wnt signaling is believed to function in the most immature cluster of cells within the crypt. A recent study, using transgenic mice, of the soluble canonical Wnt inhibitor Dickkopf1 (Dkk1), has revealed that downregulation of canonical Wnt signaling in the intestine results in decreased proliferation and dysregulated lineage development in the intestinal epithelia.[22] These functions of Wnt signaling in the intestinal epithelia raise the possibility that regulating Wnt signaling may be used to accelerate the regeneration of damaged epithelia by stimulating stem-cell function and inducing rapid expansion of the progenitor cells to replace the damaged surface by functional epithelial cells. However, Wnt signaling seems to be closely related to the cell proliferation of immature cells, and we must be aware of cancer development when we think of modulating Wnt signaling for the treatment of benign diseases. For this purpose, we must know more of the function of Wnt within the intestinal epithelial cells and make clear what makes the difference between normal epithelial development and carcinogenesis when Wnt signaling is constitutively active in an immature intestinal epithelial cell.

Regulating differentiation of the intestinal epithelia by Notch signaling

Another common signaling pathway used in the development of various tissues is now known to feature in the development of intestinal epithelial cells. Based on gene knockout studies, various transcription factors downstream of Notch signaling have been revealed to show depletion of intestinal epithelial cells in a lineage-specific manner[23-26] (Table 1). These transcription factors seem to regulate binary cell fate decision by suppressing or enhancing the expression of cell lineage-specific genes. In contrast with Wnt signaling, so far Notch signaling seems to have little involvement in the maintenance of stem-cell properties, but, rather, plays a critical role in the cell fate decision during the maturation of epithelial cells. Various basic-Helix–Loop-Helix type transcription factors are the downstream effectors of Notch signaling, and among these factors, Hes-1 and Math-1 are the major factors that are shown to be involved in intestinal epithelial cell development. Hes-1 seems to drive progenitor cells to an absorptive-type cell fate, whereas Math-1 seems to drive progenitor cells towards a secretory-type cell fate.[23,24,27] Little is known about the target genes of these transcription factors, but analyzing the genes regulated by these factors may reveal the precise mechanism determining the decision between absorptive cells and secretory-type cells. Further study of the role of Notch signaling may reveal the reason why some patients with severe inflammatory bowel diseases show difficulty in the regeneration of the

Table 1. Phenotypes of mice deficient in basic helix-loop-helix (bHLH) type transcription factor downstream of the Notch signaling pathway

Gene	Phenotype in the intestine	Reference no.
Hes1	Increase of secretory lineage cells in the small intestine	23
Math-1	Decrease of secretory lineage cells in the small intestine	24
Neurogenin-3	Decrease of neuroendocrine cells in the small intestine	25
BETA2/neuroD	Decrease of serotonin- or cholecystokinin-producing neuroendocrine cells in the small intestine	26

damaged epithelia. Some features of these diseases suggest that dysregulation of lineage decisions in epithelial cells is implicated in the pathogenesis of the disease; i.e., goblet cell depletion and Paneth cell metaplasia in ulcerative colitis patients. Analyzing whether these patients have dysregulation of Notch signaling molecules may lead to a regenerative therapy to induce the required lineage of cells; for example, by administering soluble Notch signaling modulators. However, as in Wnt signaling, modification of Notch signaling itself may lead to de-novo carcinogenesis,[28] so we might need a more fine-tuned way of targeting a specific molecule to make use of this signaling pathway.

Bone marrow-derived cells as a source of intestinal epithelial cells

Besides utilizing endogenous, tissue-specific stem cells as a source for tissue regeneration, recent studies have revealed that cells derived from other tissues might be another source for this purpose.[29,30] Many reports have raised the idea of the pluripotency of tissue-specific stem cells that might be used for the regeneration of other damaged tissues.[31] Although the pluripotency of adult stem cells is still controversial, bone marrow-derived cells are reported to have potential to contribute to tissue repair in various nonhematopoietic tissues.[32,33] Such tissues may also include the intestinal epithelial, and several studies have reported the existence of intestinal epithelial cells derived from extraintestinal or bone-marrow origin, both in mice and in humans.[34–36] Our examinations of patients with damaged gastrointestinal tract epithelia have also shown that epithelial cells of bone marrow-derived origin exist in each part of the gastrointestinal tract, but usually in extremely low populations.[37] Cell fusion has been reported to contribute to such findings,[38–42] both in vitro and in vivo. If this is the general mechanism of the observed bone marrow-derived cells in the intestine, the use of bone marrow cells as a therapeutic source may be limited,[43] but this issue still remains controversial,[44–47] and so far there is no evidence that rapid cell fusion occurs in the intestinal epithelia. Whether or not these observations of bone marrow-derived cells are the results of cell fusion, the possibility that bone marrow-derived cells contribute to some part of tissue construction in the intestinal epithelia still remains to be pursued.

Bone marrow-derived cells in the regeneration of the gastrointestinal epithelia

Whether bone marrow cells could be used as an alternative source for intestinal epithelial regeneration is yet to be proved, but several lines of evidence suggest that these cells may be one of the candidates.[48] In humans, bone marrow-derived epithelial cells contributed to the regeneration of the damaged epithelia after graft-versus-host disease (GVHD).[37] Also, the contribution of bone marrow-derived epithelial cells was observed to accelerate regeneration in the epithelia of gastric ulcer.[37] This was the first report showing clinical evidence that bone marrow cells might be a source of epithelial regeneration in humans. In mice, similar results have been reported, using experimental colitis.[49] Other reports suggest that bone marrow cells could contribute as myofibrobasts that support epithelial cells in the crypt region, and that these bone marrow-derived myofibrobasts are increased during repair after epithelial damage.[50,51] These reports support the strategy of using bone marrow cells for regeneration of damaged intestinal epithelia. However, there are reports suggesting that the contribution of bone marrow cells in the intestinal epithelia is rare.[46,52] This finding may due to the fraction of bone marrow cells used for transplantation, the degree of tissue damage, or the technical procedure used to detect donor-derived cells,[53] and further precise study is needed.

Possible clinical applications of bone marrow-derived cells

The clinical use of bone marrow-derived cells for the regeneration of the intestinal epithelia might aim to incorporate the cells in the treatment of patients with refractory inflammatory bowel disease. These patients suffer from continuous, untreatable ulcers, resulting in resection of the gastrointestinal tract.[8,54] To treat these patients using bone marrow-derived cells, we raise two hypothetical strategies (Fig. 2); bone marrow transplantation, or the infusion of humoral factors to further accelerate the contribution of bone marrow-derived cells to the regeneration of the gastrointestinal epithelia.[48]

Several case reports have suggested that allogenic or autologous bone marrow transplantation occasionally has some ameliorating effect on ulcerative colitis or Crohn's disease.[55–58] A recent report showed that autologous bone marrow cell transplantation following intense immunosuppressive therapy induced long-term remission in patients with refractory Crohn's disease.[59] The effect of bone marrow transplantation in these patients is believed to be the elimination of pathogenic lymphocytes from the inflammation site by the reconstitution of hematopoietic cells. From the former results, another effect, that the transplanted bone marrow cells also contributed to epithelial regeneration, could be considered. Adding enriched bone marrow-derived cells that have the potential to contribute to epithelial regeneration,

when tissue damage has occurred, might lead to accelerated regeneration of the epithelia (Fig. 2A). At present, there is no evidence that bone marrow-derived epithelial cells contribute to the regeneration of the gastrointestinal tract in these patients. Also, several problems must be solved to establish bone marrow transplantation as a regenerative therapy for the gastrointestinal epithelia; i.e., which fraction of the bone marrow is the critical one required, whether we should use cells of autologous or allogenic origin, and whether we should deliver cells by intravenous injection or by other routes.

Another approach to the use of bone marrow cells for the regeneration of the intestinal epithelia is to infuse humoral factors into the gastrointestinal epithelia, to further increase the contribution of bone marrow cells. Recently, the injection of granulocyte-macrophage colony-stimulating factor (GM-CSF), a stimulating factor of hematopoietic progenitor cells, has been shown to have an improving effect in patients with refractory Crohn's disease.[60,61] Experimental colitis in mice was also ameliorated by the injection of GM-CSF.[62] The precise mechanism as to why this was effective is unclear, but this factor may have stimulated the bone marrow and introduced these cells into the damaged epithelia. These lines of clinical and experimental evidence provide a basis for the use of GM-CSF for the regeneration of damaged epithelia in inflammatory bowel diseases. Many other humoral factors are now being studied to determine whether they have regenerative effects on the intestinal epithelia, but the data cited here suggest that factors stimulating hematopoietic-lineage cells might be one of the candidates (Fig. 2B).

In conclusion, utilization of bone marrow cells for the regeneration of the intestinal epithelia needs much understanding of the molecular mechanism of how bone marrow-derived epithelial cells are generated, and how bone marrow transplantation or GM-CSF treatment exert their effects in patients with inflammatory bowel disease.

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Hyperexpression of Inducible Costimulator and Its Contribution on Lamina Propria T Cells in Inflammatory Bowel Disease

TOSHIRO SATO,* TAKANORI KANAI,† MAMORU WATANABE,† ATSUSHI SAKURABA,* SUSUMU OKAMOTO,* TAKAAKI NAKAI,* AKIRA OKAZAWA,* NAGAMU INOUE,* TERUJI TOTSUKA,† MOTOMI YAMAZAKI,† RICHARD A. KROCZEK,§ TSUNEO FUKUSHIMA,‖ HIROMASA ISHII,* and TOSHIFUMI HIBI*,¶

*Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan; †Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan; §Molecular Immunology, Robert-Köch Institute, Berlin, Germany; ‖Department of Surgery, Yokohama City Hospital, Yokohama, Japan; and ¶Center for Comprehensive and Advanced Medicine, School of Medicine, Keio University, Tokyo, Japan

Background & Aims: To investigate the role of inducible costimulator (ICOS), a new member of the CD28 family involved in regulation of T-cell activation and chronic intestinal inflammation, we assessed its expression and functional role in patients with inflammatory bowel disease (IBD). _Methods:_ Expression of ICOS, CD28, and cytotoxic T-lymphocyte antigen (CTLA) 4 on intestinal lamina propria mononuclear cells (LPMC) from patients with ulcerative colitis (UC), Crohn's disease (CD), and normal controls was determined using flow cytometry and immunohistochemistry. Expressions of the ICOS ligand, B7h, on lamina propria B cells, macrophages, and epithelial cells (EC) in the intestinal mucosa were also determined using flow cytometry. The functional costimulatory effect of ICOS on LPMC was assessed by the proliferative response and cytokine production. _Results:_ $CD4^+$ LPMC expressing ICOS was significantly increased in the inflamed mucosa of IBD patients but not in inflammatory or normal controls. B7h was also significantly up-regulated on B cells, macrophages, and EC in inflamed mucosa of IBD patients. Proliferative responses of anti-CD3/ICOS costimulation were significantly higher compared with those of anti-CD3 monoclonal antibody (mAb) alone. Anti-CD3/ICOS-stimulated-LPMC from UC secreted significantly increased amounts of interleukin (IL)-5 among the 3 groups. In contrast, anti-CD3/ICOS-stimulated-LPMC from CD secreted significantly increased amounts of interferon (IFN)-γ in the presence of IL-12. _Conclusions:_ Highly expressed ICOS in activated $CD4^+$ LPMC of IBD patients contributes to the dysregulated immune responses in IBD. Because ICOS hyperexpression was limited to inflammatory sites in IBD patients, ICOS would be a feasible therapeutic target for the treatment of IBD.

Crohn's disease (CD) and ulcerative colitis (UC) are the 2 major forms of chronic inflammatory bowel disease (IBD). Although their etiopathology remains unknown, increasing evidence has outlined that immune mechanisms play an important role in their pathogenesis.[1–3] These include increased T-cell infiltration in inflamed mucosa and abnormal cytokine production by lamina propria (LP) T cells. In both UC and CD, a cytokine imbalance has been postulated as playing an important role in the initiation and/or perpetuation of intestinal inflammation.[4] T helper (Th)-1 cytokines (interferon [IFN]-γ, interleukin [IL]-12, IL-18) predominate in CD, Th2 cytokines (IL-5) tend to predominate in UC, and activated effector T cells in inflamed mucosa from both diseases have been implicated in their pathogenesis.[5–8]

In the activation of naïve T cells, 2 signals are required from antigen-presenting cells (APC) for optimal activation of antigen-specific T cells.[9,10] The first signal is provided by the specific antigen recognition through the interaction of major histocompatibility complexes (MHC) and the T-cell receptor (TCR)-CD3 complex. The second signal (called a costimulatory signal) is delivered to T cells by costimulatory molecules expressed on APC. Even if T cells receive an adequate TCR signal, they fail to respond effectively and are rendered anergic or undergo apoptosis in the absence of costimulation.[11] The most studied costimulatory signal is that of CD28, which resides on the T cell and responds to its counter receptors, B7-1 (CD80) and B7-2 (CD86), on APC.[12,13]

Abbreviations used in this paper: APC, antigen-presenting cell; CD, Crohn's disease; CTLA, cytotoxic T-lymphocyte antigen; EC, epithelial cells; IBD, inflammatory bowel disease; ICOS, inducible costimulator; LPMC, lamina propria mononuclear cells; mAb, monoclonal antibody; MLR, mixed lymphocyte reaction; NL, normal control; PBMC, peripheral blood mononuclear cells; TCR, T-cell receptor; Th, T helper; UC, ulcerative colitis.

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CD28-mediated costimulation plays a critical role in T-cell activation, as evidenced by studies in animal models of experimental allergic encephalomyelitis (EAE), collagen-induced arthritis, asthma, and experimental colitis, in which the severity of disease was markedly reduced by blocking the CD28/B7 pathway.[14–17] Cytotoxic T lymphocyte antigen 4 (CTLA4), the second member of CD28 family, is another receptor for B7-1/B7-2, and its expression is rapidly up-regulated following T-cell activation. CTLA4 has a higher affinity for B7-1/B7-2 than CD28, and its engagement delivers a negative signal.[18,19] Thus, CTLA4 might inhibit T-cell responses by out-competing CD28 for binding to B7-1/B7-2, by inducing immunosuppressive cytokines, or by directly antagonizing CD28-mediated signaling.[20]

Recently, a third member of the CD28 family, the inducible costimulator (ICOS,[21] also known as AILIM[22]) has been identified. Similar to CTLA4, ICOS is also induced rapidly on T cells after T-cell engagement with APC. Conversely, ICOS enhances T-cell proliferation and characteristic cytokine secretion, high levels of IL-4, IL-10, and IFN-γ and a low level of IL-2. The function of ICOS at the effector phase is more dominant than that of CD28. This is probably because of the induction of its antagonist, CTLA-4, on activated T cells. ICOS is expressed at high levels by Th2 cells and at low levels by Th1 cells in mice.[23] In addition, ICOS is expressed on T cells in germinal centers. These studies indicate a role for ICOS in T-cell help for B cells and functional studies have confirmed this.[23,24] The ligand for ICOS, B7h[25] (also known as ICOSL,[26] B7RP-1,[27] LICOS,[28] and B7-H2[29]) has been identified and is constitutively expressed on B cells and monocytes and induced on nonlymphoid cells by the inflammatory cytokine tumor necrosis factor (TNF)-α. Furthermore, it is known that GL50, a cytoplasmic variant of B7h, exhibits binding to ICOS; however, it has been detected only in lymph nodes, and its functional differences have not yet been determined.[30]

In this study, we demonstrate (1) the hyperexpression of ICOS in LP $CD4^+$ T cells from the inflamed mucosa of IBD patients, (2) a correlation between ICOS expression and disease activities, and (3) a functional role of ICOS in IBD, including proliferative responses and cytokine production by anti-ICOS costimulation.

Materials and Methods

Patients and Samples

Mucosal samples were obtained from inflamed and uninflamed areas of intestinal mucosa of 54 patients with CD (31 surgical and 23 biopsy specimens; 40 inflamed and 14 noninflamed specimens), 46 patients with UC (28 surgical and 18 biopsy specimens; 39 inflamed and 19 noninflamed specimens). The ileum was the primary site of CD involvement in 12 patients, ileocolonic in 27, and colon in 15. As a normal control (NL), mucosal samples were obtained from macroscopically and microscopically unaffected areas of 43 colonic and ileal specimens from colon cancer patients who underwent surgery and in which histopathologic examination revealed no malignancy or inflammation. As a disease control, mucosal samples were obtained from the inflamed areas of intestinal mucosa of 10 patients with acute colitis (1 *Salmonella enteritis*, 4 amebic colitis, 2 diverticulitis, and 3 ischemic colitis). The mucosa was prepared immediately after stripping away the underlying submucosa by blunt dissection. Informed consent was obtained from all patients before obtaining samples.

Disease activity in each patient with CD was analyzed according to Crohn's Disease Activity Index (CDAI) and from endoscopic and histopathologic data. Extraintestinal manifestations had been diagnosed in 6 patients. When the experimental study was performed in patients with CD, 7 patients were receiving only steroids, 24 were receiving steroids and sulfasalazine, and 17 were receiving only sulfasalazine; 6 patients had been undergoing nonspecific therapy for the previous 3 months.

In the UC group, disease activity was defined by the Truelove–Witts criteria and endoscopic (Matts grade) and histopathologic data. When the experimental study was performed, 3 patients were receiving only steroids, 23 were receiving both steroids and sulfasalazine, and 15 were receiving only sulfasalazine; 5 patients had been undergoing nonspecific treatment for at least 3 months.

Cytokine and Antibodies

Purified phycoerythrin (PE) and fluorescein isothiocyanate (FITC)-conjugated anti-human ICOS mAb (F44, mouse IgG_1) were generated as described.[21] Purified anti-human B7h mAb (2D3, mouse IgG_{2b}) was purchased from Lab Vision (Fremont, CA). Control mouse IgG_1 mAb (MOPC-21), control mouse IgG_{2b} (G155-178), purified anti-human CD3 mAb (UCHT1, mouse IgG_1), purified anti-human CD28 mAb (CD28.2, mouse IgG_1), FITC-conjugated CD19 (HIB19, mouse IgG_1), FITC-conjugated CD33 (HIM3-4, mouse IgG_1), FITC-conjugated CD45 (HI30, mouse IgG_1), PE-conjugated anti-mouse IgG_2a+b (X57), PE-conjugated anti-human CTLA4 mAb (BNI3, mouse IgG_{2a}), PE-conjugated anti-human CD45RA mAb (HI100, mouse IgG_{2b}), PE-conjugated anti-human CD45RO mAb (UCHL1, mouse IgG_{2a}), FITC and PerCP-conjugated anti-human CD4 mAb (RPA-T4, mouse IgG_1), and FITC and PerCP-conjugated anti-human CD8 mAb (HIT8a, mouse IgG_1) were purchased from BD Pharmingen (San Diego, CA).

Immunohistochemistry

Tissue sections were stained by a well-described method.[7] Bowel specimens were placed in cold calcium- and magnesium-free phosphate-buffered saline (CMF-PBS; GIBCO-BRL, Gaithersburg, MD) at the time of surgery or colonoscopy

examination and transported immediately to the laboratory. Samples were embedded in Tissue-Tek (OCT compound; Sakura Finetechnical, Tokyo, Japan), and snap frozen in liquid nitrogen. Cryostat sections (8 μm) were fixed by microwave (500 W, 15 seconds) with distilled water containing 0.1 mol/L sodium cacodylate and 0.025% calcium chloride. Sections were incubated for 30 minutes with a blocking solution containing normal goat serum (ICN, Aurora, OH) diluted 1:10 in PBS. Control mouse IgG (4 μg/mL), mouse anti-human ICOS mAb (F44) (4 μg/mL), or mouse anti-human B7h (2D3) (4 μg/mL) was incubated for 2 hours at room temperature, followed by 2 hours incubation at room temperature with goat anti-mouse IgG coupled with FITC (1:50 dilution, ICN). The antibodies were diluted in PBS containing normal goat serum. All steps were followed by a wash in 3 changes of PBS, pH 7.4, for 5 minutes. Sections were mounted with glycerol/PBS with paraphenylenediamine, and colocalization of FITC was examined using a confocal fluorescence microscope (LSM 410 inverted laser scan microscope; Carl Zeiss, Jena, Germany).

Isolation of Lamina Propria Mononuclear Cells and Epithelial Cells From Intestinal Mucosa

Lamina propria mononuclear cells (LPMC) were isolated using enzymatic techniques as previously described.[7] Briefly, the dissected mucosa was incubated in calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS) containing 2.5% fetal bovine serum and 1 mmol/L dithiothreitol (Sigma-Aldrich, St. Louis, MO) to remove mucus. The mucosa was then incubated in a medium containing 0.75 mmol/L EDTA (Sigma-Aldrich) for 60 minutes at 37°C. During this procedure, intraepithelial lymphocytes and epithelial cells (EC) were released from the tissue, and tissues containing LPMC were collected and incubated in a medium containing 1% collagenase type III (Worthington Biochemical Corp., Freehold, NJ) for 60 minute at 37°C. The fraction was pelleted twice and resuspended in 3 mL of 40% Percoll (Pharmacia Biotech, Piscataway, NJ), which was then layered over 60% Percoll before centrifugation at 1500 rpm for 30 minutes at 18°C. Cells in the top 40%–60% layer interface contained >95% pure viable LPMC. For isolation of EC, supernatant after EDTA treatment were washed twice, pelleted, and resuspended in 3 mL of CMF-HBSS, which was then layered over 30% Percoll before centrifugation at 1500 rpm for 30 minutes at 18°C. Cells in the top CMF-HBSS/30% Percoll layer interface contained >95% pure viable EC. The purity of the resulting EC and LPMC was confirmed by flow cytometry. To isolate mucosal naïve ($CD45RA^+$) $CD4^+$ T cells and memory ($CD45RO^+$) $CD4^+$ T cells, $CD4^+$ T cells were separated from LPMC by positive selection by a magnetic cell sorting system (MACS; CD4 multi-sort kit; Myltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. $CD45RA^+$ naïve T cells or $CD45RO^+$ memory T cells were then separated from LP $CD4^+$ T cells by positive selection using the MACS.

Flow Cytometry

Flow cytometric analysis was performed as previously described.[7] Viable lymphocyte populations were gated using forward scatter/side scatter and negative staining with propidium iodide. For staining, 1×10^6 freshly isolated or cultured cells were incubated with 20 μL of 1 μg/mL PE-conjugated anti-human ICOS mAb (F44) and FITC-conjugated anti-CD4 mAb, anti-CD8 mAb, anti-CD45RA mAb, anti-CD45RO mAb, or isotype-matched mouse IgG for 20 minutes on ice. After washing, the fluorescence intensity on the cell surfaces was analyzed using a FACScan (Becton Dickinson, Mountain View, CA). To analyze the expression of B7h protein, cells were incubated with either purified anti-human B7h mAb (2D3) or control mouse IgG_2, followed by secondary staining with PE-conjugated rat anti-mouse IgG_{2a+b} (BD Pharmingen). Freshly isolated EC were gated using negative staining with propidium iodide and FITC-conjugated CD45 mAb. LP B cells were stained with FITC-conjugated CD19 mAb. LP macrophages were stained with FITC-conjugated CD33 mAb. Cells were analyzed with a FACScan as indicated.

Reverse-Transcription Polymerase Chain Reaction Analysis for Human B7h mRNA

Total RNA was isolated from 1×10^7 freshly purified epithelial cells using RNAzol (Biotex Laboratories, Houston, TX). First-strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA with oligo (dT) primer and 400 U/mL murine Moloney leukemia virus (MMLV) reverse transcriptase (Perkin Elmer, Norwalk, CT) using the Superscript Preamplification System (GIBCO BRL, Gaithersburg, MD) in 20 μL of the reaction mixture. The mixture was incubated at 42°C for 50 minutes, heated at 94°C for 5 minutes, and then quick chilled on ice. Polymerase chain reaction (PCR) was performed on equal amounts of cDNA to amplify the cDNA of B7h. The PCR reaction mixture contained 5 μL of cDNA, 5 μL of 10X PCR buffer, 1 μL of 1.25 mmol/L deoxynucleoside triphosphate, 34.5 μL of diethyl pyrocarbonate (DEPC)-water, 2 μL of 20 μmol/L 5′ and 3′ primers, and 0.5 U of Taq DNA polymerase (Perkin Elmer, Norwalk, CT). To amplify the cDNA of B7h, the amplification was conducted at 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 3 minutes. To determine the optimal number of cycles, a range of 21, 24, 27, and 30 PCR cycles were performed. Aliquots of each PCR were transferred on a 1.2% agarose/ethidium bromide gel. Optimal numbers of PCR cycles were determined as 1 or 2 cycles less than needed to overcycle the cDNA. The primers were designed to distinguish B7h and GL50, another variant of the human ICOS ligand. Human B7h specific primers were 5′ primer, CGT GTA CTG GAT CAA TAA GAC GG and 3′ primer, TGA GCT CCG GTC AAA CGT GGC C. The human GL50 specific primers were 5′ primer, CGT GTA CTG GAT CAA TAA GAC GG and 3′ primer, TCA CGA GAG CAG AAG GAG CAG GTT CC. Human glyceraldehyde 3-phos-

phate dehydrogenase (GAPDH, as a housekeeping gene) primers were 5′ primer, TGA AGG TCG GAG TCA ACG GAT TTG GT, and 3′ primer, CAT GTG GGC CAT GAG GTC CAC CAC (Mapping Amplimers; Clonetech, Palo Alto, CA). All specific primer, synthesized by the phosphoramine method using a DNA synthesizer (model 392 PCR-MATA; Applied Biosystems, Inc., Foster City, CA), were purchased from Sawady Technology (Tokyo, Japan). A 100-bp DNA ladder (GIBCO BRL) was used as a marker. The PCR products amplified by B7h and GL50 primers were size fractioned by gel electrophoresis in 2% low-melting point agarose gels and purified by phenol-chloroform extraction. The purified PCR products were then directly sequenced by a modification of the dideoxynucleotide chain-termination method and through cycle sequencing using DNA polymerase (Sequencing High Cycle; Toyobo Co., Osaka, Japan). B7h and GL50 sequences were analyzed by aligning homologous gene regions in reference to advanced BLAST searches.

Activation of T Cells by Anti-CD3 Plus Anti-ICOS

For costimulation assay, flat-bottomed 96-well microtiter plates (Iwaki, Tokyo, Japan) were coated with anti-CD3 (1 μg/mL) plus anti-CD28 mAb (10 μg/mL), anti-CD3 (1 μg/mL) plus anti-ICOS mAb (20 μg/mL), or anti-CD3 (1 μg/mL) plus control Ab (10 μg/mL) at 37°C for 3 hours. In addition, as a control, plates were coated with control Ab, anti-CD28, or anti-ICOS alone. Proliferation assays were performed by culturing purified LPMC (2×10^5/well) for 72 hours, as previously described.[7] After incubation, cells were pulsed for 12 hours with [^{3}H]-thymidine (1 μCi/well) (New England Nuclear, Boston, MA), harvested on glass fiber filters, and counted for radioactivity (in counts per minute) in a liquid scintillation system. In another set of experiments, $CD4^+$ T cells were separated from LPMC by positive selection using the MACS. Mixed lymphocyte reaction (MLR) assay was performed by culturing purified LP $CD4^+$ T cells (2×10^5/well) with various concentrations of allogeneic monocytes separated from the same donor's peripheral blood in the presence or absence of anti-B7h mAb (2D3, Lab Vision). After 5 days initiation of MLR, cells were pulsed for 12 hours with [^{3}H]-thymidine (1 μCi/well), harvested on glass fiber filters, and counted for radioactivity (in counts per minute) in a liquid scintillation system.

To detect cytokines, supernatants were collected after 60 hours of culture, and the concentrations of IL-2, IL-4, IL-5, IFN-γ, and IL-10 were determined by sandwich enzyme-linked immunosorbent assay (ELISA; R&D, Minneapolis, MN) according to the manufacturer's instructions.

Statistical Analysis

Results are expressed as mean ± SEM. Groups of data were compared using nonparametric Mann–Whitney *U* test. Statistical significance was established at $P < 0.05$.

Results

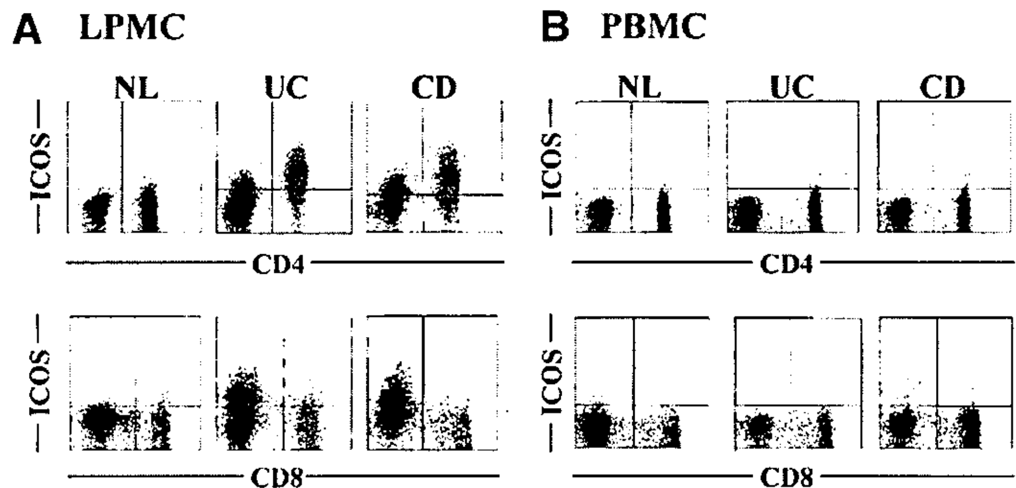
Expression of ICOS on Lamina Propria Mononuclear Cells

To assess the contribution of ICOS-B7h interactions in IBD, we first analyzed the expression of ICOS on LPMC from inflamed mucosa of IBD patients by flow cytometry. The proportions of LPMC expressing ICOS were significantly increased in inflamed mucosa from patients with UC and CD, compared with those from NL (Figure 1*A*). $ICOS^+$ cells in $CD8^+$ LPMC were detected at very low levels (Figure 1*A*), and there were no significant differences among 3 groups. In contrast, $CD4^+$ $ICOS^+$ or $CD8^+$ $ICOS^+$ cells were minimally detected in PBMC from all 3 groups (Figure 1*B*). Interestingly, surface $CTLA4^+$ $CD4^+$ LPMC were slightly, but significantly, increased in inflamed mucosa from patients with IBD, when compared with those from NL (Figure 1*C*). By contrast, CD28 was constitutively expressed at similar levels on both $CD4^+$ and $CD8^+$ LPMC from all 3 groups (Figure 1*C*). There were no differences of ICOS expression between ileal and colonic samples in CD (data not shown).

Of clinical importance, there was a significant correlation between ICOS expression on $CD4^+$ LPMC and disease activity. The percentages of $CD4^+ICOS^+$ LPMC were significantly higher in inflamed mucosa from patients with UC (49.3% ± 12.3%) and CD (47.9% ± 15.8%) than those in noninflamed mucosa (22.1% ± 3.2%; 24.0% ± 12.1%, respectively) (Figure 2*A*). Interestingly, $CD4^+ICOS^+$ cells were slightly but not significantly increased (23.3% ± 13.1%) on LPMC from patients with acute colitis, such as *Salmonella enteritis*, amebic colitis, colonic diverticulitis, and ischemic colitis when compared with NL (Figure 2*A*). For further confirmation, we next examined expression of ICOS in human IBD by confocal laser scanning microscopy. As shown in Figure 2*B*, $ICOS^+$ mononuclear cells in LP were markedly increased in inflamed mucosa from patients with UC or CD as compared with NL and non-IBD colitis controls.

Expression of B7h Molecules in Intestinal Mucosa

To investigate the surface cell phenotypes of infiltrated mononuclear cells expressing the ICOS ligand B7h, we assessed its expression in inflamed mucosa of IBD patients using confocal laser scanning microscopy. As shown in Figure 3*A*, $B7h^+$ mononuclear cells in LP were markedly increased in inflamed mucosa from patients with UC or CD as compared with NL. To determine which cells express B7h, we examined the expres-



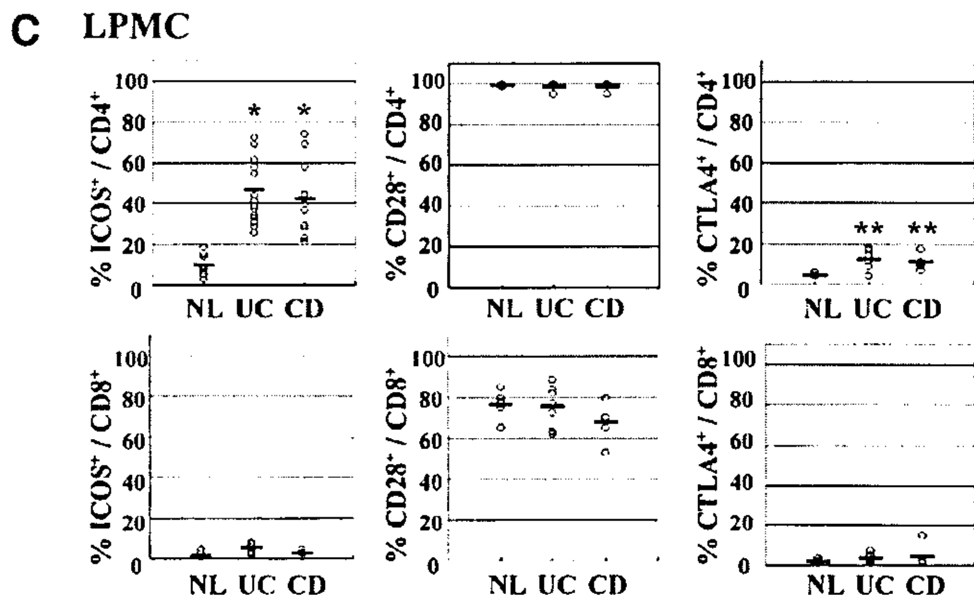


Figure 1. Expression of ICOS molecules on freshly isolated human LPMC and PBMC obtained from NL, UC, and CD patients. (*A*) Representative data showing the increased proportion of ICOS$^+$ cells in CD4$^+$ LPMC (*upper*) but not CD8$^+$ LPMC (*lower*) from UC and CD patients as compared with those from NL. (*B*) Representative data showing no expression of ICOS on CD4$^+$ PBMC (*upper*) but not CD8$^+$ PBMC (*lower*) from NL, UC, and CD patients. (*C*) Proportion of CD4$^+$ or CD8$^+$ LPMC expressing ICOS, CD28, and CTLA4 in NL (16 cases), patients with UC (21 cases), and CD (24 cases) were measured using flow cytometry. *Upper:* Proportion of CD4$^+$ LPMC expressing ICOS is significantly increased in UC and CD (*$P < 0.0005$ vs. NL). Proportion of CD4$^+$ LPMC expressing CTLA4 is slightly, but significantly, increased in UC and CD ($P < 0.005$, UC vs. NL; **$P < 0.005$, CD vs. NL). CD28 is constitutively expressed on CD4$^+$ LPMC from 3 groups. *Lower:* ICOS and CTLA4 are slightly expressed on CD8$^+$ LPMC from 3 groups. CD28 is constitutively expressed on CD8$^+$ LPMC from 3 groups. No differences are detected among the 3 groups.

sion of B7h on LPMC and EC using flow cytometry. CD19$^+$B7h$^+$ LP B cells and CD33$^+$B7h$^+$ LP macrophages were significantly increased in both UC and CD as compared with NL (B cells: NL, 41.2% ± 13.2%; UC, 67.4% ± 14.5%; CD, 68.6% ± 16.8%, respectively; NL vs. UC, $P = 0.021$; NL vs. CD, $P = 0.017$; macrophages: NL, 42.4% ± 14.5%; UC, 71.2% ± 13.3%; CD, 72.6% ± 14.7%, respectively; NL vs. UC, $P = 0.013$; NL vs. CD, $P = 0.014$). B7h was slightly but significantly up-regulated on EC from UC and CD as compared with NL (NL, 0.8% ± 1.1%; UC, 6.1% ± 3.1%; CD, 6.4% ± 2.9%; NL vs. UC, $P = 0.022$; NL vs. CD, $P = 0.019$) (Figure 3*B*). To confirm the slight but significant up-regulation of B7h in EC, we examined B7h mRNA transcripts using RT-PCR. Again, B7h mRNA was significantly up-regulated in EC from UC and CD as compared with that from NL. Furthermore, lymph node-specific GL50, a splice variant of the B7h cytoplasmic domain, was not amplified in EC from all 3 groups (Figure 3*C*).

Costimulation of T-Cell Proliferation by ICOS

To determine whether ICOS has functional costimulatory activity on LPMC, we stimulated freshly isolated LPMC from the 3 groups with anti-ICOS, anti-CD28, or control IgG in the presence or absence of anti-CD3, and, thereafter, T-cell proliferative response was determined by measuring the incorporation of [^{3}H]-thymidine. As shown in Figure 4*A*, control IgG, anti-ICOS, or anti-CD28 alone in the absence of anti-CD3 stimulation did not enhance proliferation of LPMC from all 3 groups. In contrast, anti-ICOS or anti-CD28 in the presence of anti-CD3 enhanced proliferation of LPMC from the 3 groups, as compared with stimulation by anti-CD3/control IgG (Figure 4). Consistent with a pre-

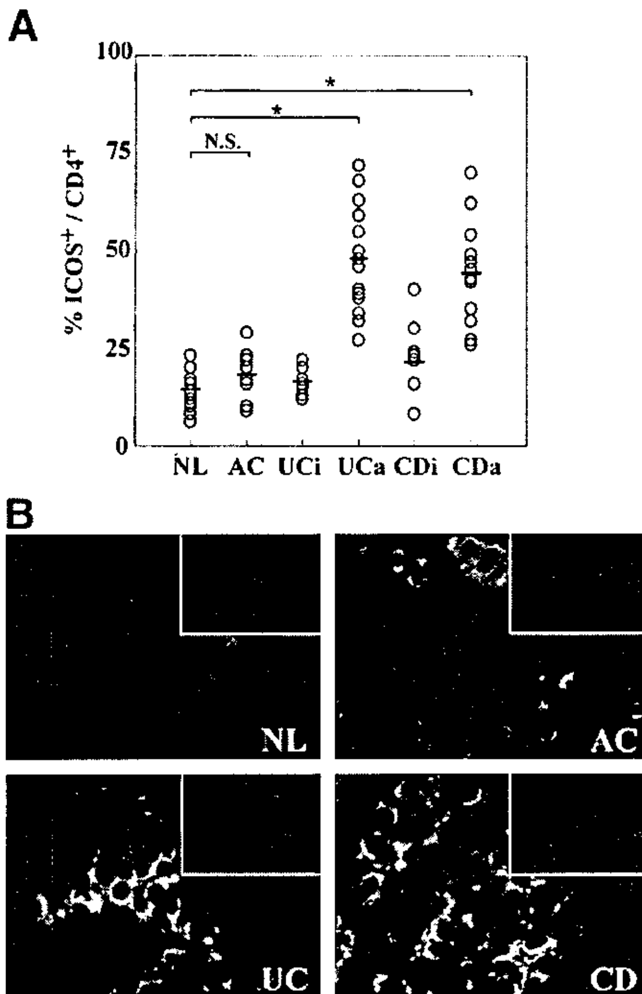


Figure 2. (*A*) Correlation between the proportion of $CD4^+$ LPMC expressing ICOS and disease activity determined by endoscopic criteria (Matts grade) in UC inactive (UCi; Matts grade = 1) and UC active (UCa; Matts grade > 3) or CDAI in CD inactive (CDi; CDAI < 150) and CD active (CDa; CDAI > 150). The proportion of $CD4^+$LPMC expressing ICOS is significantly increased in UCa ($*P < 0.0005$) and CDa ($*P < 0.0005$), compared with UCi and CDi, respectively. As disease controls, the proportion of $CD4^+$LPMC expressing ICOS in acute colitis (AC) is shown. (*B*) Confocal imaging of mucosa from NL, UC, CD, and ischemic colitis as a disease control stained with anti-ICOS mAb. *Insets* show imaging stained by control IgG.

vious report,[6] proliferative responses of LPMC from patients with UC and CD were overall significantly lower than those from NL when stimulated with anti-CD3 in the presence or absence of anti-ICOS or anti-CD28 costimulation. To further determine whether ICOS-B7h interaction affects activation of LPMC, we next conducted MLR assay. As shown in Figure 4*B*, LP $CD4^+$ T cells in the absence of allogenic monocytes (as APC) showed minimal proliferation, which was not inhibited by neutralizing anti-B7h antibody. In contrast, LP $CD4^+$ T cells stimulated by APC proliferated in a dose-dependent manner. The proliferative responses of LP $CD4^+$ T cells were significantly reduced when ICOS-B7h interaction was blocked by anti-B7h antibody, indicating the involvement of ICOS-B7h interaction in MLR. However, a similar inhibitory ability of anti-B7h to MLR was observed in all 3 groups, suggesting that ICOS could be immediately induced on NL LP $CD4^+$ T cells in MLR.

Induction of ICOS Expression on LPMC

To evaluate the kinetics of ICOS expression, LPMC from IBD patients and NL were subsequently activated with anti-CD3, and ICOS expression was determined using flow cytometry. As noted before (Figure 1*A*), ICOS expression was very low before stimuli on LP $CD4^+$ T cells from NL (10.5% ± 3.3% positive on $CD4^+$ cells) but was strongly induced by stimulation with anti-CD3 for 48 hours (84.5% ± 8.3% positive on $CD4^+$ T cells). In contrast, the levels of ICOS expression on LP $CD4^+$ T cells from IBD patients before stimuli were significantly higher than those from NL (UC: 60.3% ± 13.3% [vs. NL, $P < 0.0005$], CD: 50.3% ± 12.7% [vs. NL, $P < 0.0005$] positive on $CD4^+$ T cells). However, ICOS expression on $CD4^+$ LPMC from IBD patients after anti-CD3 stimulation was significantly lower compared with activated $CD4^+$ LPMC from NL after stimuli (UC: 54.3% ± 13.3% [vs. NL, $P < 0.05$], CD: 62.3% ± 12.7% [vs. NL, $P < 0.05$] positive on $CD4^+$ T cells) (Figure 5*A*).

Based on this reversed expression of ICOS after anti-CD3 stimulation between IBD patients and NL, we questioned whether the lower induction of ICOS on LPMC from IBD patients was due to different expressions of naïve/memory T-cell phenotypes. To this end, ICOS induction kinetics were determined using sorted naïve ($CD45RA^+$) or memory ($CD45RO^+$) LP $CD4^+$ T cells. As previously reported, most of the LP $CD4^+$ T cells from all 3 groups have the $CD45RO^+CD45RA^-$ phenotype.[31] Before anti-CD3 stimulation, ICOS was preferentially expressed on parts of $CD45RO^+$, but not $CD45RA^+$ LP $CD4^+$ T cells from all 3 groups, although the level was significantly higher on $CD45RO^+$ LP $CD4^+$ T cells from IBD patients as compared with those from NL (Figure 5*B*). $CD45RA^+$ LP $CD4^+$ T cells from the 3 groups similarly expressed ICOS promptly after anti-CD3 stimulation (Figure 5*C*). Similarly, ICOS was promptly up-regulated on $CD45RO^+$ T cells from NL after anti-CD3 stimulation (Figure 5*C*). In contrast, the expression of ICOS after anti-CD3 stimulation on $CD45RO^+$ LP $CD4^+$ T cells from IBD patients was similar compared with that before anti-CD3 mAb stimulation at any time point (Figure 5*C*).