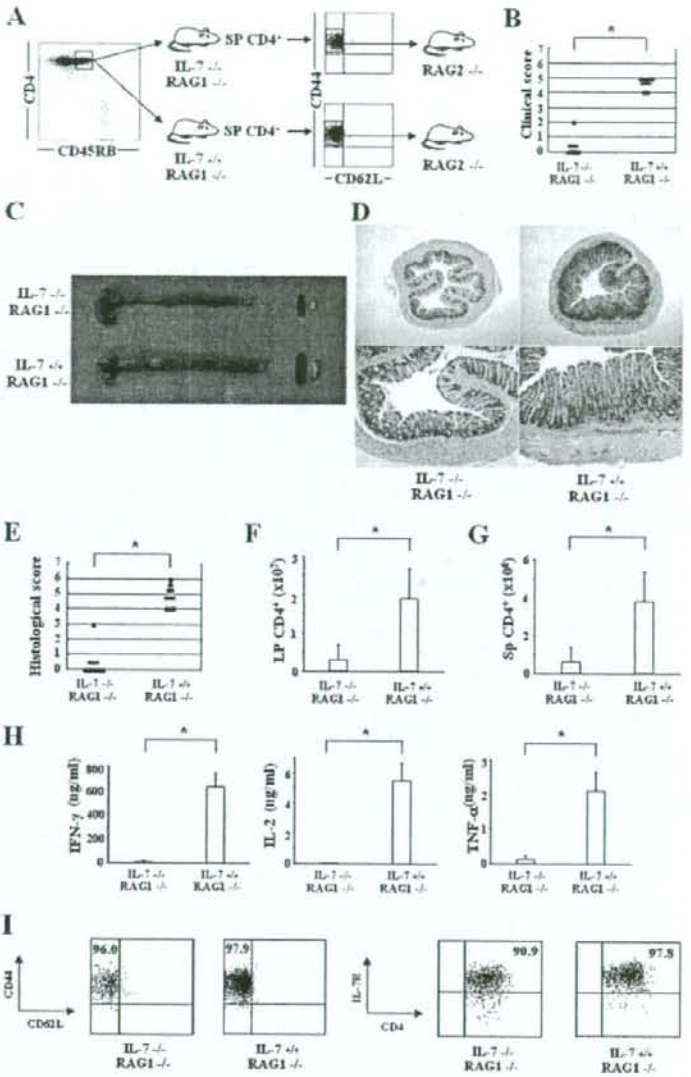

FIGURE 7. Sustained $CD4^+$ T cells in IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients do not have a potential to induce colitis when transferred to IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients. *A*, IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice were transferred with splenic $CD4^+$ T cells obtained from colitic $CD4^+$ $CD45RB^{high}$ T cell-transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (IL-$7^{+/+}$→IL-$7^{+/+}$, $n = 7$) and noncolitic $CD4^+CD45RB^{high}$ T cell-transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice (IL-$7^{-/-}$→IL-$7^{+/+}$, $n = 7$). *B*, Clinical scores were determined 4 wk after transfer as described in *Materials and Methods*. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.0005$. *C*, Gross appearance of the colon, spleen, and mesenteric lymph nodes from IL-$7^{-/-}$→IL-$7^{+/+}$ (*top*) and IL-$7^{+/+}$→IL-$7^{+/+}$ (*bottom*) mice 9 wk after transfer. *D*, Histological examination of the colon from IL-$7^{-/-}$→IL-$7^{+/+}$ and IL-$7^{+/+}$→IL-$7^{+/+}$ mice 9 wk after transfer. Original magnification: ×40 (*upper*); ×100 (*lower*). *E*, Histological scoring of IL-$7^{-/-}$→IL-$7^{+/+}$ and IL-$7^{+/+}$→IL-$7^{+/+}$ mice 4 wk after transfer. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.001$. LP (*F*) and spleen (*G*) $CD4^+$ T cells were isolated from IL-$7^{-/-}$→IL-$7^{+/+}$ and IL-$7^{+/+}$→IL-$7^{+/+}$ mice 4 wk after transfer, and the number of $CD4^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of seven mice in each group. LP, *, $p < 0.005$; spleen, *, $p < 0.05$. *H*, Cytokine production by LP $CD4^+$ T cells. $CD4^+$ LPL were isolated from each mouse at 4 wk after transfer and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. IFN-γ, IL-2, and TNF-α concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean ± SD of seven mice in each group. *, $p < 0.005$. *I*, Phenotypic characterization of LP $CD4^+$ T cells isolated from IL-$7^{-/-}$→IL-$7^{+/+}$ and IL-$7^{+/+}$→IL-$7^{+/+}$ mice 9 wk after transfer.

have divided over eight times (19) as is in a similar manner with IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients, rapid-dividing area of IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients was markedly decreased as compared with that of IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients, indicating that rapid-proliferating cells in IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients were subjected to undergo apoptosis or the rate of rapid-proliferating cells in IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients were significantly faster as undetectable as for cells divided over eight times (19) by this CSFE method.

Sustained $CD4^+$ T cells in IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients do not have a potential to induce colitis when transferred to IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients

Finally, we address a question whether sustained $CD4^+CD44^{high}$ $CD62L^-$ effector-memory type of T cells in IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with $CD4^+CD45RB^{high}$ T cells (Fig. 1) have a potential to induce colitis if they were transferred to new IL-7-competent IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice. Because it was very important to assess a possibility that a small but substantial number of $CD4^+$ T cells would be maintained by other factors, such as commensal bacterial Ag-driven TCR signaling and IL-15, as suggested by others (16, 23–25) in $CD4^+CD45RB^{high}$ T cell-transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice, but not enough to expand to induce colitis due to the absence of IL-7, we isolated splenic $CD4^+$ T cells from colitic $CD4^+CD45RB^{high}$ T cell-transferred colitic IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice and noncolitic IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice at 8 wk after transfer (Fig. 7*A*). We next retransferred these splenic $CD4^+$ T cells into new IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (Fig. 7*A*). Expectedly and similarly with the result from the adoptive transfer of colitic LP $CD4^+$ T cells (Fig. 4), IL-$7^{+/+}$ × RAG-$1^{-/-}$

recipients transferred with splenic $CD4^+$ T cells from colitic $CD4^+$ $CD45RB^{high}$ T cell-transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ (IL-$7^{+/+}$→IL-$7^{+/+}$) mice developed a severe colitis until 4–6 wk after the transfer, characterized by significant weight loss, diarrhea, and higher total clinical scores (Fig. 7*B*) and thickening of the colonic wall with inflammation (Fig. 7, *C* and *D*). In contrast, IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipient (IL-$7^{-/-}$→IL-$7^{+/+}$) mice appeared healthy and did not exhibit any signs of colitis until 9 wk after transfer (Fig. 7*B*), and no apparent thickening of the colonic wall (Fig. 7*C*). No evident pathological changes were observed in the colon (Fig. 7*D*). Average histological scores characterized by severe inflammation and epithelial hyperplasia (Fig.7*D*) were 4.90 ± 0.87 in those IL-$7^{+/+}$→IL-$7^{+/+}$ mice in contrast to 0.42 ± 1.13 in those IL-$7^{-/-}$→IL-$7^{+/+}$ mice ($p < 0.001$) (Fig. 7*E*). The average recovered numbers of LP and splenic $CD4^+$ T cells from colitic IL-$7^{+/+}$→IL-$7^{+/+}$ mice were 190.8 ± 77.3 × 10^5 cells/colon (Fig. 7*F*) and 31.3 ± 18.3 × 10^5 cells/spleen (Fig. 7*G*), respectively, whereas those from noncolitic IL-$7^{-/-}$→IL-$7^{+/+}$ mice were 29.7 ± 39.9 × 10^5 cells/colon (Fig. 7*F*) ($p < 0.005$) and 6.23 ± 13.68 × 10^5 cells/spleen (Fig. 7*G*) ($p < 0.05$), respectively. As shown in Fig. 7*H*, LP $CD4^+$ T cells from noncolitic IL-$7^{-/-}$→IL-$7^{+/+}$ mice produced significantly less IFN-γ, IL-2, and TNF-α as compared with those from colitic IL-$7^{+/+}$→IL-$7^{+/+}$ mice (Fig. 7*H*). Furthermore, flow cytometry analysis showed that the LP $CD4^+$ T cells isolated from both colitic IL-$7^{+/+}$→IL-$7^{+/+}$ recipients and noncolitic IL-$7^{-/-}$→IL-$7^{+/+}$ mice were sustained the phenotype of $CD44^{high}CD62L^-$IL-$7R\alpha^{high}$ T_{EM} cells (Fig. 7*I*).

Discussion

A central pursuit in the field of chronic immune-mediated diseases, such as IBD, has been to identify the specific factors that are responsible for the persistence of the diseases. In this study, we demonstrated that IL-7 is essential for the development and the persistence of chronic colitis by a series of adoptive transfer of normal $CD4^+CD45RB^{high}$ T cells and colitogenic LP $CD4^+$ $CD44^{high}CD62L^-$ T_{EM} donor cells into IL-$7^{+/+}$ × RAG-$2^{-/-}$ and IL-$7^{-/-}$ × RAG-$2^{-/-}$ recipients. Although rapidly proliferative responses of donor colitogenic LP $CD4^+$ T_{EM} cells was observed in IL-$7^{-/-}$ × RAG-$2^{-/-}$ recipients to a similar extent of those in recipient IL-$7^{+/+}$ × RAG-$2^{-/-}$ mice after transfer, expression of Bcl-2 was significantly down-modulated in LP $CD4^+$ T cells, and the number of recovered LP $CD4^+$ T cells was markedly decreased in the IL-$7^{-/-}$ × RAG-$2^{-/-}$ recipients as compared with IL-$7^{+/+}$ × RAG-$2^{-/-}$ recipients. These results suggest that IL-7 is critical for the persistence of chronic colitis as a survival factor for colitogenic $CD4^+$ T_{EM} cells rather than proliferative factor to sustain the intestinal inflammation.

We have previously shown a potential role for IL-7/IL-7R-mediated immune responses in intestinal inflammation. First, IL-7 transgenic mice developed chronic colitis that mimicked histopathological characteristics of human IBD (12). As chronic colitis developed, IL-7 transgenic mice showed significant infiltration of IL-$7R^{high}CD4^+$ T cells in the colonic LP. Second, we clarified that mucosal IL-$7R^{high}CD4^+$ T cells in colitic TCRα-deficient mice are the pathogenic T cells that can induce chronic colitis by the adoptive transfer of these cells into syngeneic immunodeficient RAG-$2^{-/-}$ mice, and the selective elimination of IL-$7R^{high}CD4^+$ T cells by administrating toxin-conjugated anti-IL-7Rα mAb completely ameliorated colitis (13). Third, in vitro stimulation by IL-7 enhanced the significant proliferative responses and the survival of colitic LP $CD4^+$, but not normal LP $CD4^+$, T cells (26). These previous results suggest that IL-7 might be a crucial factor for the development of chronic colitis and prompted us to investigate to prove it directly using the adoptive transfer system in the completely IL-7-deficient condition. Because adult IL-$7^{-/-}$ mice are highly lymphopenic in the peripheral blood and lymphoid organs due to the defected lymphopoiesis (27), it was impossible to compare wild-type mice and littermate IL-$7^{-/-}$ mice in terms of disease susceptibility. To overcome this issue, we generated littermate IL-$7^{+/+}$ × RAG-$1^{-/-}$ and IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice and used as recipients for the adoptive transfer of $CD4^+CD45RB^{high}$ T cells or the colitogenic LP $CD4^+CD44^{high}CD62L^-$ T_{EM} cells into these mice. Importantly, because IL-7 is not detected in lymphocytes, the present adoptive transfer system could provide a clue whether IL-7 is essential for the development and the persistence of chronic colitis.

In this study, we found that IL-$7^{-/-}$ × RAG-$1^{-/-}$ transferred with $CD4^+CD45RB^{high}$ T cells never developed chronic colitis 8 wk after the transfer (Fig. 1) and even 20 wk after the transfer (data not shown). The results showed that IL-7 is essentially needed to develop colitis in terms of disease susceptibility in this model, but it was still unclear whether IL-7 is critical for the initiation of T cell activation or the persistence of colitogenic $CD4^+$ T_{EM} cells. To clarify this issue in detail, we next conducted another adoptive transfer experiment using colitogenic LP $CD4^+CD44^{high}CD62L^-$ T_{EM} cells into IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice and IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice without the impact of T cell priming, activation, and differentiation of naive $CD4^+$ T cells. Again, we found that IL-$7^{-/-}$ × RAG-$1^{-/-}$ transferred with the colitogenic LP $CD4^+$ T_{EM} cells never developed chronic colitis after transfer (Fig. 2) in contrast to the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice that developed severe colitis. The results showed that IL-7 is especially essential for the persistence of colitogenic $CD4^+$ T_{EM} cells.

Of note, however, de Latour et al. (28) very recently reported that IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with $CD4^+$ $CD45RB^{high}$ T cells developed a wasting disease and colitis at 6 wk after transfer that was performed by very similar protocol of ours albeit to less inflammatory severity as compared with those in IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients. However, the discrepancy between their result and ours was not surprising because they used recipients that were colonized by *Helicobacter hepaticus*, a bacteria known to be associated with colitis in immunodeficient mice, such as Rag-deficient and SCID mice, indicating that their result might be due to the activated innate immune responses induced by mucosal *H. hepaticus* infection, resulting in increasing production of signal 3 cytokines, such as IL-12, type I IFNs (IFN-αβ), and type II IFN (IFN-γ), to promote expansion and survival of colitogenic effector and memory T cells (29). Because we demonstrated that the small but substantial number of memory-type of mucosal $CD4^+$ T cells were resided even in $CD4^+CD45RB^{high}$ T cell-transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$, it is likely that *H. hepaticus*-induced activation of innate immunity in their setting might have accelerated the development of *H. hepaticus*-mediated T cell expansive colitis or just T cell-independent innate immune-mediated colitis by increasing activated macrophages and granulocytes. Although we performed the specific PCR for *Helicobacter* species, including *H. hepaticus* using stool samples from mice in our facility, all data were all negative for *Helicobacter* species (data not shown). Further studies will be needed this issue.

Somewhat at odds, however, we found that rapid proliferation of donor $CD4^+$ T_{EM} cells was observed after transfer of CFSE-labeled colitogenic LP $CD4^+$ T_{EM} cells into IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice as well as into IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (Fig. 4), although the total number of recovered $CD4^+$ T cells from IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice was markedly decreased as compared with that from IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (Fig. 3). Consistent with these results, we found that Bcl-2 expression was significantly decreased

and conversely the ratio of annexin V$^+$ cells in rapidly proliferating CFSE$^-$CD4$^+$ cells was significantly increased in CD4$^+$ T cells from the transferred IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice as compared with those from the transferred IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice. These results suggest that IL-7 is not required for rapid proliferation of colitogenic LP CD4$^+$ T_{EM} cells in the lymphopenic condition but is critical for the survival of colitogenic CD4$^+$ T_{EM} cells, followed by the essential contribution for the persistent colitis. Furthermore, the kinetics study to assess an early effector phase showed no colonic inflammation at 1 and 2 wk after transfer of CD4$^+$ CD45RB^{high} T cells into the IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients and thus suggests that chronic persistent colitis is not induced by only the expansion of effector cells, but what may be needed is the continuous conversion to T_{EM} and the equilibrium between effector cells and T_{EM} cells to maintain the diseases. Another possibility, which we do not favor, is that IL-7 might be essential for the maintenance of the colitogenic LP effector CD4$^+$ T cells to sustain the disease.

It should be discussed this colitis model induced by the adoptive transfer into lymphopenic mice from the standpoint of homeostatic regulation of T lymphocytes. Conditions present in congenital mutant mice have been exploited for many years as an animal model for chronic wasting IBD, which occur several weeks after adoptive transfer of syngeneic naive CD4$^+$CD45RB^{high} T cells in the condition, which lacks regulatory T cells (14, 15). Chronic colitis results from secretion of large amounts of inflammatory cytokines, especially IFN-γ and TNF-α, by infiltrated LP CD4$^+$ cells that are chronically activated presumably by the bacterial Ags in the colon (30). The essential role of enteric bacteria is affirmed by the fact that intestinal inflammation cannot be induced if the mutant mice are reared under germfree conditions (15), indicating that enteric bacterial Ags might be responsible for the expansion of colitogenic CD4$^+$ T cells. Apart from this model, it is now well accepted that the transfer of naive CD4$^+$ T cells into a lymphocyte-deficient environment initiates proliferative responses (5, 21, 22). Careful analysis reveals that some of the transferred cells proliferate rapidly and undergo robust differentiation to memory cells, a process designated "rapid proliferation" responding to external Ags, including enteric bacteria, and other cells proliferate relatively slowly, designated "slow proliferation" responding self-Ags (21, 22). Min et al. (19) recently demonstrated that rapid proliferation of T cells is IL-7 independent, whereas slow proliferation is IL-7 dependent. Although the mechanism of our colitis model induced by the adoptive transfer of CD4$^+$CD45RB^{high} T cells would fit with enteric bacteria-inducing rapid proliferation model because rapid proliferation of donor LP CD4$^+$ T_{EM} cells was observed into IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice (Fig. 4), we found that IL-7 is critically required for the development and the persistence of colitis in mice transferred with CD4$^+$CD45RB^{high} T cells or the colitogenic CD4$^+$ T_{EM} cells. The discrepancy may be due to the difference of IL-7 dependency between the rapid proliferation, which is IL-7 independent, and the following survival step of CD4$^+$ T_{EM} cells, which is IL-7 dependent. In other words, IL-7 may be critically needed to survive the colitogenic CD4$^+$ T_{EM} cells after their rapid proliferation in the lymphopenic condition. However, it should be also noted that rapid proliferation in antibiotics-treated mice, regardless of IL-7$^{-/-}$ × RAG-1$^{-/-}$ and IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice, could be not fully abolished (Fig. 7). This indicates that other environmental Ags, such as food and bedding and self-Ags themselves, might be involved in rapid proliferation in the lymphopenic condition.

Such characteristics of our colitis model raise another important question whether the colitogenic CD4$^+$CD44^{high}CD62L$^-$ T cells can be defined as T_{EM} cells rather than effector T cells in the presence of Ags, in this case, intestinal bacteria. In general, immunological memory has evolved to warrant rapid and efficient elimination of microbial agents that repeatedly enter the organism. As a rule, immunological memory builds up, following successful elimination from the organism. In contrast, persistence of Ag, like in chronic infectious diseases, often leads to the exhaustion of the immune response (31). In immune responses in mice with chronic colitis, the target commensal bacteria are never eliminated but persist throughout life. Thus, would the colitogenic CD4$^+$ T cells in CD4$^+$CD45RB^{high}-transferred colitis model build up memory against Ags? If so, do colitogenic memory CD4$^+$ T cells play a role in the course of chronic disease? First, we found that the colitogenic CD4$^+$ T cells highly expressed both CD44 and IL-7Rα. It is generally thought that highly expressed IL-7Rα is one of accepted memory, but not effector, T cell markers, and also IL-7Rα is down-regulated via TCR stimulation in the presence of Ags. Second, memory, but not effector, CD4$^+$ T cells are critically controlled the survival by IL-7 (3, 4). Consistent with this, we also found that the colitogenic LP CD4$^+$ T cells were markedly decreased in IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice transferred with the colitogenic CD4$^+$ T cells as compared with the transferred IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice. Collectively, these data indicate that the colitogenic CD4$^+$ T cells are T_{EM} cells rather than effector CD4$^+$ T cells. In fact, Zaph and colleagues (32) recently demonstrated that *Leishmania*-specific central memory T cells develop in the presence of parasites. Although it could be argued that IBD, including murine model of chronic colitis, are due to chronic infection (persistence) of intestinal bacteria, it is likely that host and intestinal bacteria must establish some form of long-term relationship in which the immunological rules may be somewhat different from those of a brief encounter. Thus, a hallmark of T cell-mediated immune reaction to persistently expressed commensal bacterial Ags in chronic colitis would be the continual generation of Ag-specific effector and memory T cells. Because effector T cells are short-lived cells, there must exist cellular mechanisms by which effector and memory T cells specific for persistent bacterial Ags are maintained in the colitic mice.

Taken together, IL-7 is essential for the development and the persistence of chronic colitis as a critical survival factor for colitogenic CD4$^+$ T_{EM} cells rather than proliferative factor to sustain the intestinal inflammation, suggesting that therapeutic approaches targeting IL-7/IL-7R signal pathway may be feasible in the treatment of IBD.

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Disclosures

The authors have no financial conflict of interest.

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Inflammopharmacology

Review

Role of the appendix in the pathogenesis of ulcerative colitis

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Abstract. Although human appendix has been considered as a vestigial remnant, recent observations have focused attention on the role of the appendix in the pathogenesis of ulcerative colitis (UC). Many case-control studies suggest that previous appendectomy is rare in UC patients. This inverse relation is limited to patients who undergo appendectomy before the age of 20 years. Moreover, several investigators reported the improvement of UC after appendectomy, especially in young patients. In the appendix of UC patients, the CD4/CD8 ratio is significantly increased, and the proportion of CD4+CD69+ (early activation antigen) T cells, but not of CD4+HLA-DR+ (mature activation antigen) T cells, is also significantly increased. These findings suggest that the appendix may be a priming site in the development of UC. Further studies including analysis of CD4+ and CD8+ T cells are necessary to clarify the role of the appendix in the pathogenesis of UC.

Key words: Appendix – Appendectomy – Ulcerative colitis – Activated T cell – CD4+ T cell

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Mucosal T cells

Although the pathogenesis of ulcerative colitis (UC) has not been determined, an abnormal mucosal immune response plays a major role in the development and pathophysiology of UC [12, 26]. Extensive infiltration of lymphocytes, especially CD4+ T cells [17], has been observed in the inflamed mucosa of UC patients [32]. Activated CD4+ T cells exhibit increased cytotoxic activity [28] and secrete cytokines that enhance the inflammatory state resulting in tissue injury [2, 6]. Although the triggering factor for UC is still unknown, cytokine imbalance and the production of inflammatory mediators by activated CD4+ T cells play an important role in the pathogenesis of UC. T helper type 2 (Th2) cells and their cytokines, particularly interleukin (IL)-4, have been suggested to enhance the development of UC [22].

Recently, regulatory T cells, characterized by the expression of cell surface markers CD4 and CD25, have been shown to actively suppress immune responses, and lack of regulatory T cells leads to organ-specific autoimmunity [33]. On the other hand, a subpopulation of CD8+ T cells also suppresses the response of activated CD4+ T cells and B cells through an interaction that depends on expression of the major histocompatibility complex (MHC) class Ib molecule Qa-1, the mouse homolog of human leukocyte antigen (HLA)-E [9]. However, the precise role of these regulatory T cells in UC remains unclear.

Several studies concerning T cell subsets in the resected appendix have been performed previously [11], but very few have focused on the activation status of the immune cells in the appendix as well as in the uninflamed mucosa. Recent investigations including TCR-α deficient mice colitis models suggest that non-pathogenic enteric bacterial flora may be involved in the induction of

colitis [15, 30]. However, it is unclear which part of the colon is involved in priming luminal antigens as the inductive site.

CD4/CD8 ratio

We investigated the CD4/CD8 ratio in the inflamed and uninflamed colonic mucosa, especially in the appendiceal mucosa, of UC patients in order to clarify the role of the appendix in the development of UC [14]. UC patients were divided into 5 groups according to the activity and extent of the disease: active pancolitis (A-Pan), active left-sided colitis (A-Lt), A-Lt with appendiceal involvement (A-Lt/Ap), inactive pancolitis (I-Pan), and inactive left-sided colitis (I-Lt).

The CD4/CD8 ratio in the appendix significantly increased both in A-Lt and A-Lt/Ap compared with that in controls. The ratio in the appendix also tended to increase in A-Pan compared with that in controls. Interestingly, as the CD4/CD8 ratio in the appendix increased, the ratio in the rectum tended to increase, suggesting that some relations might be present in the immune responses between the appendix and the rectum.

In the normal appearance transverse colon of A-Lt/Ap, the CD4/CD8 ratio significantly increased compared with that in controls. In the entire colon, the CD4/CD8 ratio tended to increase in A-Lt/Ap compared with that in A-Lt, but it was significant only in the transverse colon. Matsumoto et al. [13] also reported that the histological inflammation grade in the entire colon was higher in A-Lt/Ap than that in A-Lt. The grade was significant both in the inflamed appendiceal orifice and in the uninflamed ascending colon. The CD4/CD8 ratio therefore may represent the inflammation degree in the mucosa.

Even in the inactive UC groups, the CD4/CD8 ratio significantly increased in the rectum compared with that in controls. Most patients with inactive UC have low-grade inflammation, and it is possible that symptomatic relapse occurs only when the inflammatory process reaches a critical intensity [31]. Also, because inflammation is a continuous process, direct assessment of the level of inflammatory activity may provide a quantitative pre-symptomatic measure of imminent clinical relapse of the disease [3]. In our study, the increased CD4/CD8 ratio suggested that the significant immuno-imbalance was persistent in the inactive rectum. Because patients with inactive UC even receiving maintenance therapy are easy to relapse [3], we suspect that the disease can relapse when the immuno-imbalance is persistent in the rectum.

Activated T cells

We also investigated the proportion of early and late activated CD4+ T cells in colonic mucosa of UC patients with CD69 as an early activation antigen and HLA-DR as a late activation antigen, respectively [14]. In the appendix, the proportion of CD4+CD69+ T cells significantly increased in all UC groups, even in the inactive UC groups, compared with that in controls. In the transverse colon, the proportion did not significantly increase in any UC groups compared with that in controls. In the rectum, the proportion significantly increased only in A-Pan, but not in the other groups, compared with that in controls. The proportion of CD4+HLA-DR+ T cells significantly increased only in the rectum of A-Pan, but not in the other areas of any groups compared with that in controls. These findings suggest that the appendix may be a priming site in the development of UC.

In TCR-α deficient mice, the pathological T cells are initially concentrated in the appendix [29]. Mucosal TCRαβ+ T cells, including CD4+ T cells, in IL-2 deficient mice appear in the colon prior to the manifestation of colitis [28]. An increase of identical T cell clones involved in the development of inflammation is detectable in the uninflamed appendix and the inflamed colon of UC patients as well as in TCR-α deficient mice [16, 21]. Therefore, the increased CD4+CD69+ T cells indicate that CD4+ T cells may be initially activated in the appendix, and may re-circulate to the entire colon and rectum (increased CD4/CD8) prior to the manifestation of UC, and inflammation originating from the rectum extends to the entire colon. The reason why the inflammation begins in the rectum is unknown.

Previous appendectomy

Although human appendix is considered as a vestigial remnant [20], recent observations have focused attention on the role of the appendix in the pathogenesis of UC. Many case-control studies suggest that previous appendectomy is rare in UC patients [1, 24, 25], raising the possibility that appendectomy protects against the subsequent development of UC [4, 18, 23, 27]. Patients with previous appendectomy also have a delayed onset of UC [23, 27], a reduced need for immuno-suppression therapy and proctocolectomy [4, 23], and a reduced relapse rate and extent of UC [18].

Previous appendectomy for an inflammatory condition (appendicitis or lymphadenitis), but not for nonspecific abdominal pain, is associated with a low risk of subsequent UC [1]. The findings suggest that the inflammatory condition preceding the appendectomy, rather than the appendectomy itself, is inversely related to the subsequent development of UC. This inverse relation is limited to patients who undergo appendectomy before the age of 20 years. Although these findings support that the appendix may be related to the pathogenesis of UC, the immunological role of human appendix is unknown.

Appendectomy in experimental model

Appendectomy in T-cell receptor (TCR)-α deficient mice, which spontaneously develop colitis resembling human ulcerative colitis at 24–30 weeks of age, suppresses the development of experimental colitis [15]. When the mice underwent appendectomy at a young age (3–5 weeks), the number of mesenteric lymph node cells at 6–7 months were markedly less than in the sham-operated mice. Furthermore, appendectomy at a young age, but not at older age (>6 weeks), suppressed the development of the colitis. These

results suggest that appendix is the priming site of cells involved in the disease process, and plays an important role in the development of colitis in the mice.

Therapeutic appendectomy

We first reported the improvement of UC (A-Lt/Ap) without medication during the 3 years after appendectomy in a young patient (21-year-old), and proposed that appendectomy may have a place as a therapeutic strategy in UC patients [19]. Järnerot et al. [10] also performed laparoscopic appendectomy in 6 patients with refractory UC (2 A-Pan and 4 A-Lt), and found that one young patient (26-year-old) was in remission with continued maintenance treatment, but 5 patients (mean age; 50.8 years, range; 44–56 years) had relapse of the disease. Histological analysis of the resected appendix showed mucosal erosions and moderate infiltrations of CD4+ T cells in our patient [19], but did not show any inflammation in all patients as reported by Järnerot et al. [10]. They concluded that appendectomy does not influence the course of established UC in a consistent way [10], which supports our results in the study [14].

Eri et al. [5] also reported the clinical course of 6 patients (mean age; 30.5 years) with refractory UC (5 A-Lt and 1 A-Lt/A) after laparoscopic appendectomy, and found that 5 patients were in complete clinical remission, and one patient had improved. Histological analysis of the resected appendix showed colitis-type inflammation (ulcerative appendicitis), containing a highly activated lymphocyte population, in the 5 patients. Recently, Jo et al. [11] reported the clinical course of 9 patients (mean age; 32.5 years, range; 13–48 years) with mildly activated UC (4 A-Pan and 5 A-Lt) after appendectomy, and found that 2 A-Lt patients with ulcerative appendicitis had improved, but the disease remained active in the other patients (3 A-Lt without ulcerative appendicitis and 4 A-Pan).

Hallas et al. [7] reported the nationwide study with complete follow-up of 202 patients (mean age; 43.3 years) with UC who underwent appendectomy after their onset of UC, and concluded that appendectomy has no beneficial effect on admission rates in UC patients. Although appendectomy is associated with a low risk for subsequent UC only in young patients [5, 10, 11, 19], especially before the age of 20 years [1], no stratification of data for any age had been performed [7]. Later, Hallas et al. [8] supported that appendectomy would be useful against UC in young subjects by analyzing those who underwent appendectomy before the age of 30 years. These findings and our results [14] indicate that appendectomy may be performed in young UC patients with ulcerative appendicitis.

Conclusion

In conclusion, appendectomy may be a benefit therapy in young UC patients with ulcerative appendicitis. Apart from the rectum, the appendix may be a priming site in the development of UC, and should no longer be considered an evolutionary redundancy. Further studies including analysis of CD4+ and CD8+ T cells are necessary to clarify the role of the appendix in the pathogenesis of UC.

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Development of consensus statements for the diagnosis and management of intestinal Behçet's disease using a modified Delphi approach

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Background. Although intestinal Behçet's disease has been treated anecdotally with various therapeutic modalities, clinical evidence regarding management of intestinal Behçet's disease is lacking. The objective of this study was to develop consensus-based practice guidelines for diagnosis and treatment of intestinal Behçet's disease by using a modified Delphi approach. ***Methods.*** Three groups of Japanese gastroenterology specialists were involved in the study: moderators, an expert panel, and a professional group. Clinical statements for ratings were extracted from relevant literature, a survey of the professional group, and by discussion among the expert panel. The expert panel rated the clinical statements according to a nine point scale. After the first round of ratings, a panelist meeting was held to discuss areas of disagreement and to clarify areas of uncertainty. The list of clinical statements was revised after the panelist meeting, and a second round of rating was conducted. ***Results.*** Thirty-two relevant articles were selected in a literature search, and 35 clinical statements were extracted. An additional 209 clinical statements were developed from the survey and discussion among gastroenterology specialists. In the first and second rounds, 56% and 60% of statements, respectively, received median scores ≥7. The range of scores decreased considerably from the first to the second round. ***Conclusions.*** 5-Aminosalycylic acid, corticosteroids, immunosuppressants, enteral nutrition, total parenteral nutrition, and surgical therapy were considered standard therapy for intestinal Behçet's disease. Infliximab, colchicines, thalidomide, other pharmacological therapy, endoscopic therapy, and leukocytapheresis were deemed experimental therapy. Based on a two-round modified Delphi approach, practice guidelines for diagnosis and treatment of intestinal Behçet's disease were developed.

Key words: intestinal Behçet's disease, consensus statements, Delphi approach

Introduction

Behçet's disease is a chronic relapsing disease with multiorgan system involvement characterized clinically by oral and genital aphthae, cutaneous lesions, and ophthalmologic, neurologic, or gastrointestinal manifestations (or some combination of these).[1] The etiology of the disease remains unknown, but many consider it among the systemic vasculitides. Although Behçet's disease occurs worldwide, the highest prevalence is reported in Japan, the Middle East, and the Mediterranean region, and it is relatively uncommon in northern Europe and the United States.[2] Various pharmacological agents have been used to treat Behçet's disease. These include colchicines, azathioprine, and corticoste-

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roids for eye involvement, colchicines and corticosteroids for arthritis, and colchicines, topical and systemic corticosteroids, thalidomide, or immunosuppressive agents for aphthous ulcers.[1,3] About 3%–16% of patients with Behçet's disease have gastrointestinal tract involvement according to several reports published in the 1990s.[4] Gastrointestinal disease typically affects the ileocecal area, although involvement of the esophagus and small intestine has been reported.[4] The most common gastrointestinal symptoms are abdominal pain, diarrhea, and bleeding. Deep ulcers are responsible for the most common intestinal complications such as severe bleeding and perforation.[5] 5-Aminosalicylic acid (5-ASA), systemic corticosteroids, and immunosuppressive agents have been used anecdotally to treat intestinal Behçet's disease. Clinical evidence regarding management of intestinal Behçet's disease, however, is very limited. Furthermore, there is no consensus for management of intestinal Behçet's disease to date. Owing to lack of clinical evidence and consensus for management of intestinal Behçet's disease, management of the disease can vary from physician to physician.

The modified Delphi approach is a consensus method that involves the administration of two or more rounds of questionnaires[6,7] that was developed at RAND in the 1950s as a tool to predict the future and applied to political-military, technological, and economics topics.[6] Unlike the original Delphi method, the modified Delphi method provides panelists with the opportunity to discuss their judgments between the ratings' rounds. It has also been used in various areas of medicine to develop consensus. This method ensures anonymity; hence, more reliable and unbiased expert opinions can be obtained. When clinical evidence is lacking and management of the disease relies mostly on expert opinion, this method is suitable for the development of guideline statements.

The objective of the study was to develop consensus-based practice guidelines for diagnosis and management of intestinal Behçet's disease.

Methods

An overview of the study

The study consisted of four phases. In brief, in the first phase, specific clinical statements for rating were extracted from the literature, from results of a survey sent to gastroenterology specialists, including gastroenterologists, a pediatric gastroenterologist, and gastrointestinal surgeons, and by discussion among members of an expert panel. These clinical statements included, but were not limited to, statements about the diagnosis of intestinal Behçet's disease, about medications and other therapy, and about dosage regimes, including doses appropriate for active disease and maintenance doses. During the second phase, the expert panelists individually rated these clinical statements for appropriateness. In the third phase, the panelists met and discussed areas of disagreement and areas of uncertainty. The panelists also discussed and revised some of the clinical statements. During the fourth phase, the revised clinical statements were rated again.

Ratings of appropriate methods for management of intestinal Behçet's disease were developed using a modified Delphi approach.[6] For the present study, a ten-member panel composed of gastroenterologists ($n = 6$), gastrointestinal surgeons ($n = 2$), and rheumatologists ($n = 2$) was established. Two of the panel members were also hospital directors, who were expected to rate the clinical statements from the viewpoint of hospital administration. The remaining eight panelists worked in academic settings. In addition to the expert panel, moderators and a professional group were involved in the study. The moderators comprised three gastroenterologists and one internist who was also familiar with epidemiology and the modified Delphi approach. The moderators searched and reviewed the literature, collected clinical statements for rating from the literature and the survey of the professional group, facilitated the panelist meetings, and analyzed the data obtained using the modified Delphi approach. The professional group (Appendix 1) was selected from the members of the Japanese Inflammatory Bowel Disease Research Group supported by the Japanese Ministry of Health, Labour and Welfare. The professional group comprised gastroenterologists, a pediatric gastroenterologist, and gastrointestinal surgeons who worked at referral centers and had had the opportunity to manage patients with intestinal Behçet's disease as well as ulcerative colitis and Crohn's disease.

Collection of clinical statements for the modified Delphi approach

Literature relevant to intestinal Behçet's disease was searched by the moderators. A computerized bibliographic database (PubMed) and a secondary database were searched to identify English-language articles published between 1980 and 2003. Initially, articles pertaining to the diagnosis and treatment of intestinal Behçet's disease were searched. The number of those articles, however, was very limited. Therefore, articles were accepted for review if they pertained to mucocutaneous or intestinal lesions of Behçet's disease.

Because information regarding the diagnosis and treatment of intestinal Behçet's disease from the literature was limited, further clinical statements were extracted from the results of the survey of the profes-

sional group. The moderators developed hypothetical clinical scenarios and questionnaires (open-ended questions) to supplement clinical statements for ratings (Appendix 2). Thirty-nine gastroenterology specialists (gastroenterologists, gastrointestinal surgeons, and a pediatric gastroenterologist) were sent clinical scenarios of intestinal Behçet's disease and a questionnaire regarding the diagnosis and management of intestinal Behçet's disease. They were asked to base their replies to the survey upon their own daily practice. Because the survey did not cover all clinical aspects of Behçet's disease, the expert panel conducted a password-protected online discussion of various topics, including classification of disease severity and treatments such as corticosteroids, immunosuppressants, total parenteral nutrition, and elemental diet, to supplement the literature search and survey results.

Modified Delphi approach

Panelists were asked to rate the appropriateness of the clinical statements regarding the diagnosis and management of intestinal Behçet's disease in view of health benefits and expected costs. Rating was on a 9 point scale, with 1 being highly inappropriate and 9 being highly appropriate. A clinical statement receiving a median score >7 from the panel was regarded as valid. A clinical statement with a median score of 7 and a range (maximum score—minimum score) of ≤4 was regarded as possibly valid, pending the panelist discussion. Clinical statements with a median score of 7 and a range of 5 or more, or a median of 6 to 6.5, were also discussed by the panelists.

After the first round of rating, panelists met to discuss areas of disagreement and to clarify areas of uncertainty. The clinical statements to be discussed were determined according to the criteria mentioned above. After the meeting, the list of clinical statements was revised. Revised clinical statements were sent to the panelists for a second round of ratings. The panelists were also informed of the results (median, maximum, and minimum scores) of the first round of ratings. No attempt was made to force the panel to reach consensus. The two-round process was designed to sort out whether discrepant ratings were due to real clinical disagreement over the use of the procedure ("real" disagreement) or to fatigue or misunderstanding ("artificial" disagreement).[6]

Results

From the literature search, 32 relevant articles were selected and reviewed (Appendix 3), and 35 clinical statements on the diagnosis and management of intestinal Behçet's disease were extracted from these articles. Most relevant articles were case reports or case series, while some were randomized controlled trials or controlled clinical trials. An additional 209 clinical statements were developed from the survey results and by discussion among the gastroenterology specialists. Seventeen of 39 professional group members (43.5%) responded to the survey, and 244 statements were rated by the expert panelists in the first round. The response rate for the first round was 100%. The result of the first round of ratings is shown in Fig. 1. A panelist meeting was held on 20 December 2003 in Tokyo. Clinical statements with a median score of 7 and a range ≥5 or those with a median of 6.5 or 6 were discussed at the meeting. The rating results of the clinical statements regarding

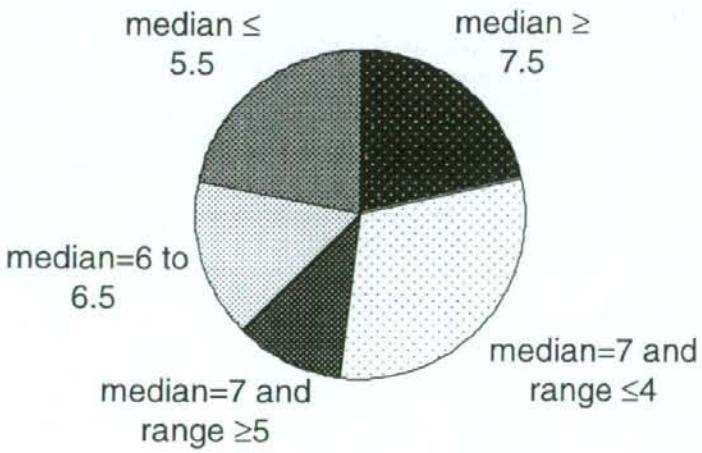

Fig. 1. Results of the first-round ratings. The proportions of the clinical statements are shown according to the median and range of their ratings. Statements with a median rating of 7 and range of 5 or more and those with a median rating of 6 or 6.5 were discussed at the consensus meeting

Table 1. Results of the first-round ratings regarding treatment of intestinal Behçet's disease

Therapy	Median > 7	Median = 7, range ≤ 4	Median = 7, range ≥ 5	Median = 6–6.5	Median < 6
5-ASA	0	4	3	7	1
Corticosteroids	5	10	12	9	3
Immunosuppressants	0	8	1	5	3
Elementary diet	2	10	1	5	3
TPN	2	0	4	2	1
Surgical therapy	4	1	1	8	6
Infliximab	0	0	2	2	0
Colchicine	0	1	0	4	1
Thalidomide	0	0	0	1	4
Other	0	0	0	3	12
Endoscopic therapy	0	1	0	6	1
Leukocytapheresis	2	5	1	0	0

Values are the number of clinical statements
5-ASA, 5-aminosalicylic acid; TPN, total parenteral nutrition

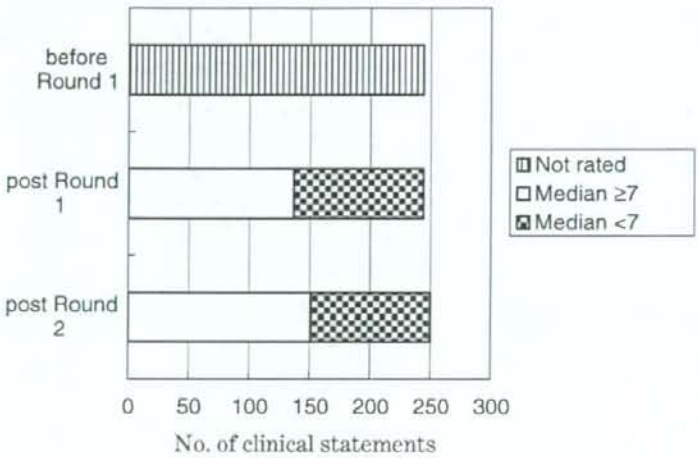

Fig. 2. Changes in median scores from the first to the second round. Because the list of clinical statements was revised after the consensus meeting, the number of clinical statements differs between the first and second round

treatment are shown in Table 1. On the basis of the results of ratings and discussion among the gastroenterology specialists at the meeting, 5-ASA, corticosteroids, immunosuppressants, enteral nutrition, total parenteral nutrition (TPN), and surgical therapy were deemed "standard therapy" for intestinal Behçet's disease. Infliximab, colchicines, thalidomide, other pharmacological therapy such as antibiotics, interferon, or combination therapy, endoscopic therapy, including ethanol spraying and leukocytapheresis, were considered "experimental therapy" for intestinal Behçet's disease. Although ratings of the clinical statements regarding leukocytapheresis were relatively high, this treatment is not widely available in Japan and is considered "experimental."

After the panelist meeting, the list of clinical statements was revised, and 250 statements were rated for appropriateness in the second round. The response rate for the second round was also 100%. The results of the first- and second-round ratings are compared in Figs. 2 and 3. About half of the statements were agreed upon in the first round. The proportion of clinical statements with a narrow range (≤3) of scores increased from the first round to the second round, indicating that there was a convergence of the panelists' opinions from the first to the second round (Fig. 3). The final rating results are shown in Fig. 4.

Based on the two-round modified Delphi approach, guideline statements for diagnosis and management of intestinal Behçet's disease were developed (Table 2). Because available clinical evidence regarding diagnosis and management of intestinal Behçet's disease is limited, we were unable to set a recommendation level for each clinical statement.

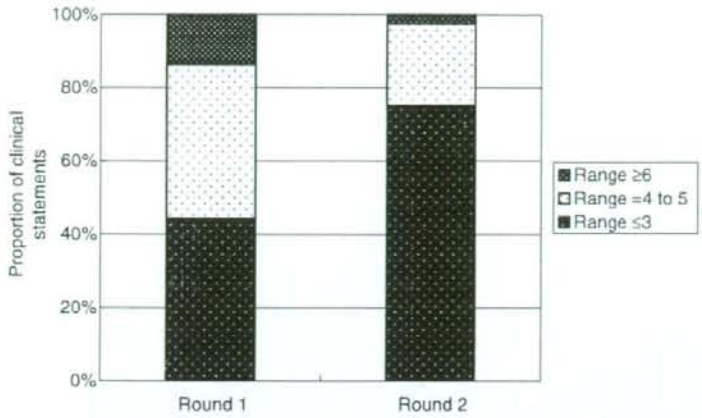

Fig. 3. Changes in the ranges of scores between the first and second rounds. The proportion of the clinical statements with a range of 3 or less was more than 70% in the second round, indicating that there was a convergence of expert opinion in the second round

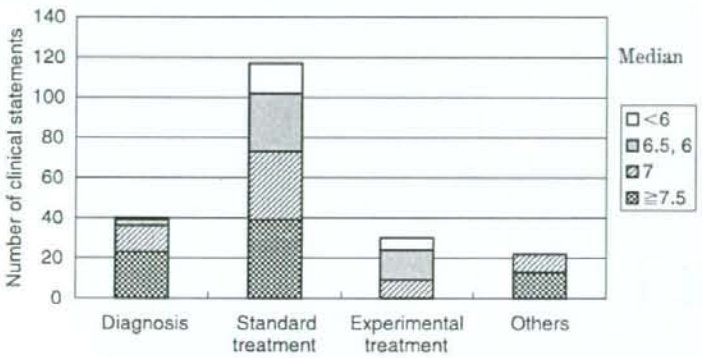

Fig. 4. Final results of ratings

Discussion

Behçet's disease involves multiple organs, including the eye, nervous system, skin, genitalia, and gastrointestinal tract. Most clinical studies of Behçet's disease published to date concern the management of mucocutaneous lesions and ophthalmologic lesions. There are no published practice guidelines to date for diagnosis and management of intestinal Behçet's disease. Even in high-prevalence areas such as Japan, the Middle East, and the Mediterranean region, intestinal Behçet's disease has been treated empirically because data from the literature regarding management of this condition are scant. The management of intestinal Behçet's disease relies heavily on expert opinion. Nevertheless, these expert opinions are not necessarily the same from one specialist to another. The consensus of expert opinion in a high-prevalence area, therefore, should be extremely helpful in daily practice.

We used the modified Delphi approach to develop practice guidelines for the diagnosis and management of intestinal Behçet's disease. The Delphi method is a consensus method and involves the administration of two or more rounds of questionnaires.[6,7] Unlike the original Delphi method, the modified Delphi method used in the present study provides panelists with the opportunity to discuss their judgments between the ratings' rounds.[6] This method has been used in various areas of medicine to develop consensus. Because it ensures anonymity, reliable and unbiased expert opinions can be obtained.

In addition to the literature search, we extracted clinical statements for ratings from results of a survey of gastroenterology specialists and by discussion among specialists. After the first round of ratings and discussion among gastroenterology experts, 5-ASA, corticosteroids, immunosuppressants, enteral nutrition, TPN, and surgical treatment were regarded as standard treat-

Table 2. Guideline statements for management of intestinal Behçet's disease

Diagnosis

1. Diagnosis of intestinal Behçet's disease can be made if
   A. There is a typical oval-shaped large ulcer in the terminal ileum, OR
   B. There are ulcerations or inflammation in the small or large intestine,
   AND clinical findings meet the diagnostic criteria of Behçet's disease
2. Tuberculosis, Crohn's disease, nonspecific colitis, drug-associated colitis and other diseases that mimic intestinal Behçet's disease should be excluded before diagnosis of intestinal Behçet's disease is made
3. Typical clinical findings of Behçet's disease such as abdominal pain, hematochezia, oral aphthae, cutaneous lesion, genital ulcer, and arthralgia should be sought. These findings may not appear at one time
4. Minimally, colonoscopy with biopsy, double-contrast barium enema, and laboratory tests are necessary to diagnose intestinal Behçet's disease. When intestinal Behçet's disease is suspected, EGD and enteroclysis are needed to determine the extent of gastrointestinal lesions
5. When intestinal Behçet's disease is suspected, dermatology, ophthalmology, orthopedics, and neurology consults are necessary
6. There is no clear classification of severity for intestinal Behçet's disease. Severity of disease is determined based on systematic symptoms, degree of abdominal symptoms, depth of intestinal ulcerations, presence of bleeding from intestinal ulcerations, laboratory findings such as WBC, C-reactive protein, and degree of anemia

Standard treatment

1. 5-ASA is indicated for all cases of intestinal Behçet's disease. The optimal dose of mesalazin for adult is 2.25–3 g/day. When sulfasalazine is used, the optimal dose is 3–4 g/day
2. Corticosteroids are indicated for patients with severe systemic symptoms, abdominal symptoms, and recurrent gastrointestinal bleeding. When 5-ASA is not effective for mild to moderate cases of intestinal Behçet's disease, corticosteroids are indicated
3. Initial dose of corticosteroids is 0.5–1 mg/kg per day of prednisolone for 1–2 weeks. When clinical improvement is observed, prednisolone should be tapered by 5 mg every week. It is advisable that >10 mg of prednisolone not be given for a long period of time
4. Clinical symptoms, C-reactive protein, and healing of ulcerations should be used as indications for tapering and discontinuation of corticosteroids
5. Immunosuppressive agents are indicated when patients are corticosteroid-dependent or corticosteroid-resistant. Azathioprine of 50–100 mg/day is the standard dose
6. EN is indicated for patients who are refractory to pharmacological therapy or have severe intestinal disease. After the patient's condition improves with TPN, EN is usually indicated
7. EN with elementary diet can be tapered and discontinued after the symptoms and signs improve. It can be continued if the patient tolerates EN well.
8. TPN is indicated for patients with severe systemic symptoms such as fever, intestinal stenosis, fistula, bleeding, or impending perforation of the intestine. It is usually used for a limited period of time
9. Surgical resection is indicated when a patient has intestinal stenosis, fistula, severe bleeding, or ileocecal lesion that is refractory to medical therapy. Resection of the intestine should be minimized. Because postoperative recurrence is common, the patients should be treated with 5-ASA or EN postoperatively

Experimental treatment

1. When a patient cannot tolerate EN and continues to have bleeding despite corticosteroids or immunosuppressive agents, infliximab can be considered as an experimental therapy
2. Colchicine is indicated for a patient with eye involvement
3. When a patient is corticosteroid-dependent or -resistant, leukocytapheresis may be used
4. Consensus could not be reached regarding other experimental therapy, including thalidomide, antibiotics, and endoscopic spray of ethanol on intestinal ulceration

EGD, esophagogastroduodenoscopy; EN, enteral nutrition

ment. Other treatments such as infliximab, colchicines, and thalidomide were considered experimental. The consensus indicates that 5-ASA is indicated for all cases of intestinal Behçet's disease. Corticosteroids are indicated for patients with moderate to severe abdominal or systematic symptoms or for patients who continue to have symptoms despite 5-ASA administration. When a patient develops stenosis, perforation, or massive hemorrhage, surgical treatment is required. Postoperatively, patients should be given either 5-ASA or enteral nutrition.

There are a few limitations in the present study. First, most of the guideline statements were based on expert opinions because there is no solid evidence regarding the diagnosis and management of intestinal Behçet's disease. Because clinical evidence regarding intestinal Behçet's disease is limited, obtaining a consensus of expert opinion in a region of high prevalence of the disease such as Japan is an alternative to clinical evidence. Because the available clinical evidence was limited and most clinical statements were based on expert opinions, we were unable to set recommendation

levels for the statements. Second, the guidelines have not been endorsed by any organizations. Therefore, the statements are a summary of opinions of Japanese experts. Third, the guidelines need to be reevaluated after their implementation in clinical practice. A prospective evaluation of the guidelines is necessary to address the effectiveness and outcome of care. Ideally, this process should be carried out by international experts. Finally, the present consensus did not cover histopathological diagnosis. This means of diagnosis should be included in the next version of the consensus.

In summary, we developed consensus-based guidelines for diagnosis and management of intestinal Behçet's disease by using a modified Delphi approach. Further research is necessary to address the impact of the guidelines on the outcome of patients with intestinal Behçet's disease.

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Appendix 1

Professional group who participated in the survey

Tsuneo Fukushima (Yokohama City Municipal Hospital), Katsuyoshi Hatakeyama (Niigata University), Nobuo Hiwatashi (Iwaki Kyouritsu Hospital), Masahiro Igarashi (Kitasato University), Mitsuo Iida (Kyushu University), Hiroaki Ito (Osaka University), Fukunori Kinjo (Ryukyu University), Atsuo Kitano (Higashi Sumiyoshi Morimoto Hospital), Kazuya Makiyama (Nagasaki University), Toshiyuki Matsui (Fukuoka University), Souichirou Miura (National Defense Medical College), Toshihiro Sakurai (Ashiya Chuo Hospital), Toshio Sawada (Gunma Cancer Center), Kenji Suzuki (Niigata University), Tsuyoshi Tomomasa (Gunma University), Toshikazu Yoshikawa (Kyoto Prefectural University of Medicine), Masahiko Watanabe (Kitasato University).

Appendix 2

Survey and questionnaire

Please indicate how you would examine and treat the following three cases. You may write about tests, treatment, and follow-up.

Case 1

A 26-year-old woman noted right lower quadrant pain 4 months ago. She also developed low-grade fever and loose stool 2–3 times a day. On physical examination, there was mild tenderness in the right lower quadrant. There were a few tender nodular erythematous lesions bilaterally in the lower legs. Laboratory test results were erythrocyte sedimentation rate (ESR) 25 mm/h, C-reactive protein (CRP) 0.45 mg/dl, and hemoglobin (Hgb) 12.8 g/dl. Other physical findings and laboratory values were unremarkable. Double-contrast barium enema and colonoscopy revealed a round-shaped ulceration in the cecum extending to the ileocecal valve. She stated that she has often developed oral aphthae and genital erosions. She denied visual disturbance.

Case 2

A 50 year-old woman presented at another hospital with abdominal pain 6 years ago. Double-contrast barium enema and colonoscopy demonstrated a deformed ileocecal valve and a deep round ulcer in the terminal ileum. She was treated with 5-ASA without resolution of abdominal pain. After she was admitted and put on NPO (nothing by mouth), her abdominal symptoms resolved. She has been taking about half of her calories by enteral nutrition since then. When she experienced exacerbation of symptoms a year ago, prednisolone 20 mg/day was begun, and she is now on 5 mg/day. Because she had intermittent abdominal pain, she visited your clinic to seek a second opinion. Imaging studies over 6 years have been reportedly unchanged. She has had recurrent oral aphthae, but no skin lesions, genital ulcerations, or eye symptoms. Laboratory test results were significant for ERS, 46 mm/h, CRP, 0.88 mg/dl, and Hgb, 11.4 g/dl.

Medical therapy	Indication	Specific dose and treatment duration	How to evaluate the effectiveness of treatment?
Corticosteroids			
5-aminosalicylic acid			
Immunosuppressant			
Elementary diet			
Total parenteral nutrition			
Thalidomide			
Other			

Surgical therapy (specify)	Indication	Medications given post-operatively	How to evaluate the effectiveness of treatment?

Case 3

An 18 year-old man presented with fever, abdominal pain, diarrhea, and hematochezia. Colonoscopy revealed multiple punched-out ulcerations in the terminal ileum and ascending and transverse colon. There were multiple small ulcers and erosions in the descending and sigmoid colon. He was treated with 5-ASA and prednisolone 30 mg/day with improvement. Upon tapering of prednisolone to 5–10 mg/day, his diarrhea and hematochezia recurred. He has been on prednisolone 10 mg/day for the past year. He cannot tolerate enteral nutrition.

Diagnosis

What are the characteristic findings of intestinal Behçet's disease? Please give some examples of clinical and laboratory findings.

Selection of treatment option

Please comment on the following treatments for intestinal Behçet's disease. If an option is not on the list, please add it to the list and include a comment.

Appendix 3

Selected articles for review

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A diacylglycerol kinase inhibitor, R59022, stimulates glucose transport through a MKK3/6-p38 signaling pathway in skeletal muscle cells

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Abstract

Diacylglycerol kinase (DGK) is one of lipid-regulating enzymes, catalyzes phosphorylation of diacylglycerol to phosphatidic acid. Because skeletal muscle, a major insulin-target organ for glucose disposal, expresses DGK, we investigated in the present study a role of DGK on glucose transport in skeletal muscle cells. PCR study showed that C2C12 myotubes expressed DGKα, δ, ε, ζ, or θ isoform mRNA. R59022, a specific inhibitor of DGK, significantly increased glucose transport, p38 and MKK3/6 activation in C2C12 myotubes. The R59022-induced glucose transport was blocked by SB203580, a specific p38 inhibitor. In contrast, R59022 failed to stimulate both possible known mechanisms to enhance glucose transport, an IRS1–PI3K–Akt pathway, muscle contraction signaling or GLUT1 and 4 expression. All these results suggest that DGK may play a role in glucose transport in the skeletal muscle cells through modulating a MKK3/6-p38 signaling pathway.
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Keywords: Diacylglycerol kinase; Glucose transport; Skeletal muscle cell; p38; R59022

Lipid-regulating enzymes involve the glucose metabolism. For instance, acyl CoA:diacylglycerol acyltransferase 1 knockout mice exhibited increased insulin sensitivity [1]. Moreover, mitochondrial acyl-CoA:glycerol-sn-3-phosphate acyltransferase 1 knockout mice exhibited increased hepatic insulin sensitivity [2]. Thus, lipid-regulating enzymes are possible to be involved in glucose metabolism.

Diacylglycerol kinase (DGK) is one of lipid-regulating enzymes, catalyzes phosphorylation of diacylglycerol (DG) to phosphatidic acid (PA) [3–7]. Ten mammalian isoforms have been identified, and are classified into five subtypes based on their primary structure. DGKs contain a conserved catalytic domain and an array of other conserved motifs that are likely to play a role in lipid–protein and protein–protein interactions in various signaling pathways dependent on DG and/or PA production. Although DGK is known as one of lipid-regulating enzymes, little is known about the implication of DGK for glucose metabolism. Because skeletal muscle expresses DGK isoforms [3] and is a major insulin-target organ for glucose disposal [8,9], we made a hypothesis that DGK, a lipid-regulating enzyme, plays a role in glucose metabolism in the skeletal muscle. To clarify the above hypothesis, we investigated in the present study a role of DGK on glucose transport in skeletal muscle cells.

Materials and methods

Cell culture. C2C12 mouse myoblast cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in

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Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and 10% fetal bovine serum (Sigma, St. Louis, MO). Confluent myoblasts were differentiated to myotubes by lowering the serum concentrations to 5% horse serum (Invitrogen, Carlsbad, CA). Cells were used for the experiments after 4–6 days of differentiation.

Chemicals. R59022, a DGK inhibitor, was purchased from Calbiochem (San Diego, CA) and was dissolved in dimethy sulfoxide (DMSO) with a final concentration of 0.1% DMSO in culture medium. Bovine serum albumin (BSA) used was free fatty acid-free grade (Sigma). [^{3}H]2-deoxyglucose was purchased from American Radioactive Chemicals (St. Louis, MO). 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was obtained from Toronto research chemicals (North York, ON). SB203580 was purchased from LC Laboratories (Woburn, MA). Antibodies used were purchased from Cell Signaling technology (Danvers, MA), except: insulin receptor substrate-1 (IRS1) and p85 subunit of phosphatidylinositol 3-kinase (PI3K) (Upstate Biotechnology, Lake Placid, NY); phosphotyrosine-RC20 (BD Biosciences, San Jose, CA); Akt substrate of 160 kDa (AS160) (Novus Biologicals, Littleton, CO); glucose transporter (GLUT) 1 and 4 (Santa Cruz Biotechnology, Santa Cruz, CA).

RT-PCR analysis. Total RNA was extracted from C2C12 myotubes at day 6 after differentiation using TRIzol reagent (Invitrogen). RT-PCR was described previously [10]. Briefly, the amplification was carried out in a 50 μl of reaction mixture containing cDNA synthesized with oligo-dT primer (ReverTra Ace, TOYOBO, Osaka, Japan) correspondent to 50 ng of total RNA, 25 pmol each of the forward and reverse primer (except for DGKβ, 10 pmol), 0.2 mM dNTPs and 1.25 U of KOD Dash Taq DNA polymerase (TOYOBO). The reaction conditions were: 30 cycles of 94 °C for 30 s, 58 °C for 2 s and 74 °C for 30 s. The products were separated electrophoretically in a 2% agarose gel and stained with ethidium bromide. A mouse whole brain of C57Bl/6Ncrj (Charles River Japan, Tokyo, Japan) was used for positive control. Expression of 36B4 was confirmed to show the quality of cDNA. Intron-spanning primers were used in order to avoid false positive due to genomic contamination and described in Table 1.

Glucose transport assay. Glucose transport was assayed by measuring [^{3}H]2-deoxyglucose uptake as described [11]. Briefly, differentiated C2C12 myotubes (day 4–5) were treated for 24 h or indicated time with several concentrations of R59022 or vehicle (DMSO) in serum-free DMEM containing 0.2% BSA and then Krebs–Ringer-phosphate buffer containing 0.2% BSA for 40 min. For positive control, insulin (final concentration, 100 nM) was added for 15 min. Uptake of [^{3}H]2-deoxyglucose was measured for last 4 min. Uptake was measured routinely in triplicate or quadruplicate for each experiment.

Protein extraction, Immunoprecipitation, and Western blot. C2C12 myotubes were treated with several doses of R59022 or vehicle for 24 h in DMEM containing 0.2% BSA. Insulin or AICAR was used for positive control as indicated. Protein extraction and Western blotting were performed as described previously [10,12]. Briefly, total proteins were extracted with lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 10% glycerol) containing protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitors (Sigma). For the analyses of IRS1 phosphorylation and IRS1/PI3K association, 500 μg protein were immunoprecipitated with anti-IRS1 antibody using protein A/G agarose beads for 16 h at 4 °C, and then eluted using Laemmli buffer containing β-mercaptoethanol. For the other analyses, equal amount of protein (20–50 μg/lane) were resolved on 7.5 or 10% SDS–PAGE. The bands were visualized using a luminoimage analyzer (Fujifilm, Tokyo, Japan) and quantified using a MultiGauge software version 2.2 (Fujifilm).

Measurement of p38 mitogen-activated protein kinase (p38) activity. C2C12 myotubes were treated with several doses of R59022 or vehicle for 24 h and with 100 nM insulin for 10 min as a control. The activity of p38 was analyzed using an assay kit (Cat. #9820, Cell Signaling Technology) according to the manufacturer's instruction.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed by analysis of variance and subsequent Fisher's LSD test. $P < 0.05$ was considered statistically significant.

Results

First, we examined and confirmed whether C2C12 myotubes express DGK by RT-PCR. The DGK has ten isoforms (α, β, γ, δ, η, κ, ε, ζ, ι, and θ) and classified into five subtypes based on the functional domains (Type 1: DGKα, β, γ; Type 2: DGKδ, η, κ; Type 3: DGKε; Type 4: DGKζ, ι; Type 5: DGKθ). We analyzed in this study at least one isoform out of each subtype of DGK. PCR studies revealed that C2C12 myotubes expressed DGKα, δ, ε, ζ, and θ isoforms (Fig. 1A, upper panel), whereas mouse brain as a positive control expressed all isoforms tested (Fig. 1A, lower panel).

Next, we examined the effect of a DKG inhibitor [13], R59022 on glucose transport in C2C12 myotubes to determine whether DGK affects glucose transport in the skeletal muscle cells. We used C2C12 myotubes in this study because the cells express multiple DGK isoforms as demonstrated in Fig. 1A. As demonstrated in Fig. 1B, insulin as a positive control significantly stimulated glucose transport in this experimental condition and R59022 similarly increased glucose transport measured using [^{3}H]2-deoxyglucose in C2C12 myotubes. Statistical significance was observed at a dose of 10 μM of R59022 and the stimulation of glucose transport by R59022 was dose-dependent. Fig. 1C shows the time–course change of glucose transport by a 30 μM dose of R59022. Stimulation of glucose transport by R59022 started at 8 h and the stimulation was observed until at least 24 h after the inhibitor.

Table 1
Primers used for PCR

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon size (bp)
36B4	TCCTTCTTCCAGGCTTTGGG	TCCTCCGACTCTTCCTTTGC	512
DGK α	TCAGCCCAGAAGACTTCGCT	TGCCGTCCTGATCCATCTCT	495
DGK β	TTCTGGAAGCTGAGCTGCCT	TGAGTCGCCACACATGCTGT	537
DGK γ	TCATGAGGGCATACCTGGAG	TTCCCAGGAGGACCAGCAAA	600
DGK δ	TCCAGCCACCTGGGTACATT	TCTTCCTCACCAGGCTCCAA	541
DGK ε	TCCCCCTGTGCTCTTACTGT	TCTCCAGCATAGCCTGTACC	592
DGK ζ	TCTGAGGAGCAGATCCAGAG	TTGCTGGCCTTGAGGGTGTT	545
DGK ι	TTCCTGTGGCTAACGGTCCA	TGCAGCATGAAGCAGGTCAC	451
DGK θ	TCCAGCTGATTGAGGTGCTC	TAGCTCAAAGACCTGGTGCG	533

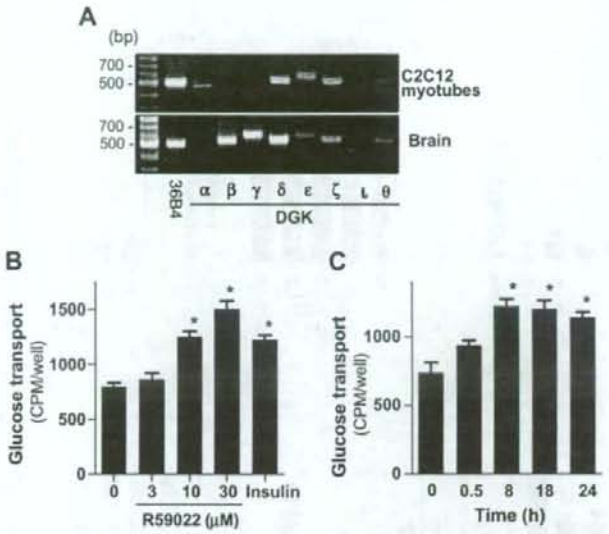

Fig. 1. (A) mRNA expression of DGK isoforms (α, β, γ, δ, ε, ζ, ι, or θ) in C2C12 myotubes (day 6) and mouse brain as a positive control was detected by RT-PCR. Dose–response effect of R59022 on glucose transport (B) and time–course change of glucose transport by R59022 in a dose of 30 μM (C) in C2C12 myotubes were shown. Insulin treatment (100 nM) was performed as a positive control. Glucose transport was assayed using [^{3}H]2-deoxyglucose for last 4 min. Data are expressed as means ± SE ($n = 3$–4). $^*P < 0.05$, when compared vehicle only.

In the next step, we attempted to clarify the mechanism by which R59022 stimulates glucose transport in C2C12 myotubes. Because it has been reported that glucose transport is deeply regulated by the IRS1–PI3K–Akt signaling pathway and the translocation of GLUT from cytosol to cell surface [14], we tested the above possibility in the increased glucose transport by R59022 in C2C12 myotubes. The effects of R59022 on phosphorylation of IRS1, association of IRS1/PI3K and phosphorylation of Akt in C2C12 myotubes were examined. As illustrated in Fig. 2A, B and C, R59022 failed to stimulate IRS1 phosphorylation, association of IRS1/PI3K or Akt phosphorylation whereas insulin as a positive control potently stimulated these three tested biochemical markers. Since muscle contraction is an another stimulator for glucose transport independently of IRS1–PI3K–Akt pathway by activating AMP-activated protein kinase (AMPK) [15], we made a hypothesis that muscle contraction signal by activating AMPK plays a role in the increased glucose transport by R59022 in myotubes. As shown in Fig. 2D, R59022 did not stimulate phosphorylation of AMPK, whereas AICAR, a chemical activator for AMPK, used as a positive control, indeed enhanced its phosphorylation in C2C12 myotubes. Because AS160 has recently been identified as an integrated down-stream mediator for Akt and AMPK towards GLUT translocation [16], we examined the effect of R59022 on AS160 phosphorylation in C2C12 myotubes. As shown in Fig. 2E, AICAR but not R59022 activated AS160 phosphorylation in C2C12 myotubes.

To test a possibility that the increased glucose transport by R59022 may be resulted from up-regulation of GLUT, we examined the effect of R59022 on GLUT expression in C2C12 myotubes. As seen in Fig. 2F and G, R59022 did not affect the protein expression of GLUT1 and 4, respectively.

It has been reported that p38 is implicated in glucose transport not by translocation of GLUT but by activation of GLUT [17]. To investigate the possible involvement of p38 activation on R59022-induced stimulation of glucose transport in the skeletal muscle cells, we examined the effect of R59022 on phosphorylation of p38 in C2C12 myotubes. As demonstrated in Fig. 3A and B, immunoblotting clearly shows that R59022 dose-dependently stimulated phosphorylation of p38 in C2C12 myotubes. A significant increase in p38 phosphorylation was obtained by R59022 at 10 μM or larger doses in C2C12 myotubes. We next investigated whether phosphorylation of p38 by R59022 is dependent upon its kinase activity using *in vitro* immunoprecipitation-kinase assay. Fig. 3C shows that R59022 increased the phosphorylation of ATF-2, the substrate of p38.

To clarify whether R59022-induced stimulation of glucose transport is indeed caused by p38 activation in C2C12 myotubes, we examined the speculation using SB203580, an inhibitor of p38. As shown in Fig. 4A, insulin or R59022 significantly increased in glucose transport in C2C12 myotubes. Although SB203580 by itself did not change glucose transport, R59022-induced stimulation of glucose transport was blocked by SB203580 in C2C12 myotubes, suggesting that activation of p38 is functionally