TABLE 5. Clinicopathologic Characteristics of UC Patients with Colorectal Cancer or Dysplasia

Patient	Age	Sex	Duration (y)	Extent	Histology	Stage	p53 Ab	p53 Staining
CA1	52	F	4	Left	Well-diff	IV	−	+++
CA2	33	M	15	Total	Well-diff	II	+	+
CA3	32	M	7	Total	Well-diff	I	−	+++
CA4	65	M	11	Total	Well-diff	0	+	+
CA5	60	M	22	Left	Well-diff	I	+	++
CA6	30	M	13	Total	Well-diff	II	+	+
CA7	37	F	10	Left	Por+Muc	IV	+	++
CA8	51	F	19	Total	Well-diff	I	+	+
HGD	47	M	22	Left	HGD	n.a.	−	−
LGD1	38	F	15	Total	LGD	n.a.	−	++
LGD2	41	F	17	Total	LGD	n.a.	+	+
LGD3	65	M	10	Left	LGD	n.a.	−	+
LGD4	50	F	25	Total	LGD	n.a.	+	−

CA, cancer; HGD, high-grade dysplasia; LGD, low-grade dysplasia; Total, pan-colitis; Left, left-sided colitis; Well-diff, well-differentiated adenocarcinoma; Por, poorly differentiated adenocarcinoma; Muc, mucinous adenocarcinoma; n.a., not applicable.

gosity (LOH) of the *p53* gene occurred in association with carcinogenesis in UC and that p53 Abs reflected the accumulation of mutated p53 protein. To date, Hammel et al[37] reported 1 p53 Ab-positive UC-associated CRC in their study of 54 CRCs, the remaining 53 patients being sporadic ones. Previous studies reported high sensitivity and specificity of p53 Abs in patients with various types of cancers.[38] In the present study we had considerably high sensitivity of p53 Abs in UC patients with CRC, although a small number of patients was analyzed. One patient with p53 Ab-negative UC-associated CRC with liver metastasis in our study actually showed positive staining for p53 in tumor tissue. Tang et al[39] reported that the frequency of low p53 Ab levels was significantly increased in Stage IV patients with metastasis because the immune system and humoral response was suppressed in these patients, which could account for our seronegative case. By lowering the cutoff value of p53 Ab titer, the positivity, and in turn, the sensitivity for CRC could increase, while the specificity and negative predictive value would decrease. Considering the existence of p53-negative UC-associated CRCs that indicate better prognoses,[36] such a cutoff value makes it impossible that the sensitivity would be 100%. In the present study the positivity in sporadic CRCs was 52.4%, which is a little higher compared with the positivity in the literature, indicating that this cutoff value would be appropriate.

The specificity of p53 Ab has been a matter of debate. Most of commercially available ELISA kits utilize wildtype p53 recombinant protein for trapping serum p53 Abs. p53 protein epitopes recognized by p53 Abs are localized in amino- and carboxy-termini of the protein, similar to those found in animals immunized with wildtype p53 protein. Hence, the immune response to p53 is directed against immunodominant epitopes unrelated to mutational hot spots. It has also been suggested that rather than mutation type per se, p53 protein accumulation due to prolonged half-life by the mutation might be a common linkage to p53 Ab production.[40] Interestingly, p53 Abs have also been found in patients having a tumor that do not present evidence of p53 protein accumulation (as seen in 1 of our patients with LGD) because of a frameshift/stop/splice mutation or in the absence of mutation.[41] The presence of p53 Ab might be due to modification in p53 antigen processing or presentation to the immune system without protein accumulation. Finally, a decrease of p53 Ab levels after resection of the tumor in the present study could support specificity of the Ab for p53 protein accumulated tumor cells, which is also demonstrated by other studies.[37,42]

p53 Abs are detected in various types of cancers, such as colorectal, breast, lung, and pancreatic cancers[38] and the frequency of p53 Abs in patients with neoplasms is usually correlated with the frequency of *p53* mutations in tumor tissue, and most patients with p53 Abs exhibit an accumulation of p53 in their neoplastic cells.[29] Therefore, serological analysis of p53 can be used as a complementary procedure with molecular and immunohistochemical methods, since it does not require tumor tissues and can be easily used for follow-up of patients with p53 alterations. In sporadic CRCs, several studies found an association between p53 Abs and short survival[43–45] and these may reflect that mutation of the *p53* gene in the remaining allele in addition to allelic loss of p53 appears to be a relatively common event in the final stages of tumorigenesis in the adenoma-carcinoma sequence. On the other hand, *p53* mutation appears to be an early event

that precedes *p53* loss of heterogeneity in the tumorigenesis of UC-associated CRC[21] and the inflammation of UC mucosa is considered to be a so-called premalignant status. p53 Abs are usually IgG, indicating a secondary response after prolonged immunization by p53 protein accumulation; thus it is reasonable to presume that such p53 Abs could be used as an early indicator of *p53* mutations in tumors in which such alterations occur early during tumoral progression. Such conditions have been known to date, i.e., lung cancer and heavy smokers, angiosarcoma of the liver and workers exposed to several carcinogens such as vinyl chloride, esophageal adenocarcinoma and Barrett's esophagus, and, moreover, CRC and UC. To our knowledge, there is only 1 prospective study that addressed the importance of p53 Abs in such individuals at high risk. Lubin et al[29,46] reported that p53 Abs were present in 2 heavy smokers prior to clinical diagnosis of lung cancer and they concluded that p53 Abs could be used as an early marker for lung cancer. Following this study, several studies have demonstrated that p53 Abs can be found in sera of high-risk individuals.[47–50] We also found p53 Abs even in sera of 12.8% of UC patients without neoplasia. We cannot exclude endoscopically undetectable neoplasms in these patients; however, positivity of serum p53 Ab is inconsistent with that of Cioffi et al's recent report[51] and none of these patients have developed CRC or dysplasia since then.

It is logical to assume that because UC-associated CRC arises in the setting of chronic inflammation, factors associated inflammation, such as oxidative stress, might well contribute to the molecular alterations seen in the tissue of inflammatory bowel disease. Hussain et al[52] showed a high frequency of *p53* mutations in inflamed tissue more than in uninflamed tissues from UC patients and concluded that increased frequency of specific *p53* mutated alleles in noncancerous UC colon tissue may confer susceptibility to the development of CRC in an inflammatory microenvironment. Therefore, it is speculated that p53 Ab-positive UC patients are at risk of developing CRCs. In accordance with other studies of sporadic CRCs,[39,53] in our series we found no correlation with demographic or clinicopathological features, except for disease duration. We found a strong correlation between p53 Ab positivity and disease duration, which is consistent with established high-risk factors for the development of UC-associated CRC.[9] In the present study we cannot conclude whether seropositivity for p53 Abs is an independent factor from disease duration for the development of CRC; however, positive serum p53 Ab might indicate risk for the development of CRC because exclusion of UC patients complicated with CRC diminished the significance of differences between the longer disease duration group and the shorter one. Considering the effectiveness of detection of CRCs in the present study, percentages of CRC patients were 2.8% in the whole UC group, 3.7% in the group of patients with extensive disease, 4.2% in the group of patients with longer disease duration (≥ 8 years), and 5.1% in the group of patients with extensive disease and longer disease duration; however, it was 14.0% in a group of patients positive for serum p53 Ab and 20.0% in a group of patients positive for serum p53 Ab with longer disease duration, while the number of patients with CRC missed in each group was similar (Fig. 2).

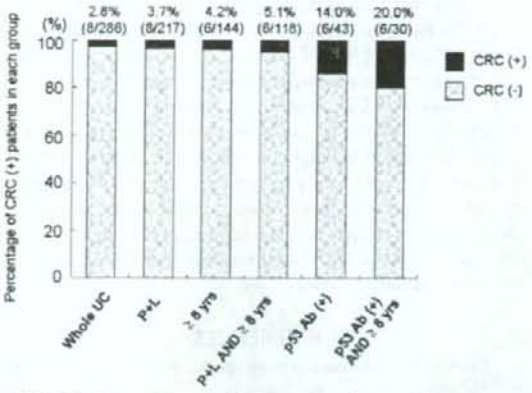

FIGURE 2. Detection rate of CRCs in certain surveillance group of ulcerative colitis (UC). Using measurement of serum p53 Ab by ELISA, the percentage positive for CRC were improved 5.1% to 14.0% or 20.0%. The number (2) of patients with CRC missed were the same in both groups. P, pan-colitis; L, left-sided colitis; CRC, colorectal cancer.

Our previous reports on 1-8U and MSI suggested that genetic events precede histological progression of UC-associated cancer and dysplasia and that the development of UC-associated neoplasms is not dependent only on the duration of inflammation, but on both the duration and severity of inflammation.[30,31] These findings may explain the fact that UC patients with chronic continuously severe inflammation develop UC-associated neoplasms in shorter periods of time than previously reported.[30] Unfortunately, analyses of MSI or 1-8U as well as immunohistochemical detection of mutated p53 protein requires tissue specimens, which is not convenient in clinical settings. Therefore, screening for serum p53 Ab by ELISA can be applied as a routine clinical procedure. However, the present study does not support the idea that p53 Ab is suitable as a primary screening tool instead of surveillance colonoscopy because of the low positive predictive value and a false-negative rate that is not negligible. To demonstrate the epidemiological usefulness of screening for serum p53 Ab in patients with UC, a decade-long prospective cohort study in patients at high risk with serial measurements of serum p53 Ab and serial colonoscopies should be done. As negligible numbers of patients drop from the program or refuse surveillance colonoscopy in clinical practice, further evaluation of serum p53 Ab in patients with UC is necessary.

In conclusion, screening for serum p53 Ab by ELISA seems to have too low a positive predictive value and sensitivity to take the place of colonoscopy as the initial screen or to soften current screening and surveillance recommendations for patients with UC; however, it deserves further evaluation to determine whether it might, in combination with colonoscopy showing minimal inflammation, be used to identify patients who could safely be left for a longer interval before repeat colonoscopy. In addition, it could be helpful for improving the effectiveness of surveillance programs by salvaging the patients at high risk who drop from the program or refuse surveillance colonoscopy.

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Curriculum vitae of intestinal intraepithelial T cells: their developmental and behavioral characteristics

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Acknowledgements
We would like to thank all members of our laboratories who have contributed to portions of our work embodied in this review paper. The data provided in the preparation of this review were supported in part by a Grant-in-Aid for Creative Scientific Research, the Japan Society for the Promotion of Science (13GS0015), by Special Coordination Funds for Promoting Science and Technology from the Japanese Ministry of Education, Culture, Sports, Science and Technology, by Research on Specific Diseases, Japanese Ministry of Health, Labor and Welfare, and by Keio University Special Grant-in-Aid for Innovative Collaborative Research Projects (T.H.). T.N. is a research fellow supported by the 21st Century Center-of-Excellence Program for Life Science from MEXT (M.S.).

Immunological Reviews 2007
Vol. 215: 154–165

Immunological Reviews
0105-2896

Summary: The alimentary tract has an epithelial layer, consisting mainly of intestinal epithelial cells (IECs), that is exposed to the exterior world through the intestinal lumen. The IEC layer contains many intestinal intraepithelial T cells (IELs), and the total number of IELs constitutes the largest population in the peripheral T-cell pool. Virtually all γδ-IELs and many αβ-IELs in the mouse small intestine are known to express CD8αα homodimers. A wide range of evidence that supports extrathymic development of these CD8αα$^+$ IELs has been collected. In addition, while several studies identified cells with precursor T-cell phenotypes within the gut epithelium, how these precursors, which are dispersed along the length of the intestine, develop into γδ-IELs and/or αβ-IELs has not been clarified. The identification of lymphoid cell aggregations named 'cryptopatches' (CPs) in the intestinal crypt lamina propria of mice as sites rich in T-cell precursors in 1996 by our research group, however, provided evidence for a central site, whereby precursor IELs could give rise to T-cell receptor-bearing IELs. In this review, we discuss the development of IELs in the intestinal mucosa and examine the possibility that CPs serve as a production site of extrathymic IELs.

Keywords: *CD8αα$^+$-IEL, γδ-IEL, αβ-IEL, extrathymic development of IEL, cryptopatches*

Introduction

Surfaces in the body in contact with the outside world include the epidermis and the mucous epithelia. Directly below both the epidermis and the mucous epithelia is an extensive basement membrane (Bm) that serves as a thin wall separating them from the interior of the body, and no capillaries or lymphatic vessels are present in the epidermis and mucous epithelia. Therefore, any lymphomyeloid cells distributed in epidermis and mucous epithelia are extravasated from the postcapillary venules in the interior of Bm, and they must move into the epidermis and mucous epithelia by crossing Bm. T cells and B cells evolved as key players in the immune system of vertebrates, and an infinite number of antigen-specific receptors are produced by a mechanism called somatic gene rearrangement. It has been known for some time that lymphocytes are distributed in the epidermis and mucous epithelia, and in about the middle of the 1970s, it became clear that most intestinal intraepithelial lymphocytes settling in the small intestine of mice are T cells [intestinal

intraepithelial T cells (IELs)] (1). Furthermore, almost all T cells in the epidermis of laboratory mice are those expressing homogenous γδ-type T-cell receptors (TCRs), also known as dendritic epidermal T cells (DETCs). The surprising finding concerning these γδ-DETCs is that they are produced in the thymus at about day 15 of embryonic life and are thus derived from the first wave of fetal γδ thymocytes (2). In this review article, we focus our discussion mainly on findings obtained in mice concerning development of IELs distributed among intestinal epithelial cells (IECs).

Surprising evidence, showed by studies using a monoclonal antibody to TCR, is that almost all mouse IELs are T cells (3–9). IELs are radically different from T cells residing in other sites of the body; most of them are ill-defined T cells with unusual but distinctive characteristics. These cells are located at the front line of defense, at the point which the interior of the body comes in contact with the greatest numbers of antigens from the exterior world.

The interior of the Bm consists of *lamina propria* (LP) that contains abundant immunoglobulin A $(IgA)^+$ B cells, $CD3^+$ T cells (Fig. 1), and various lymphomyeloid cells. In contrast (as discussed later), the exterior of the Bm contains an IEC layer with prominent colonization of CD8α-expressing T cells (Fig. 1). The marked differences between the inside and the outside of the Bm are very important in connection with clarification of *in vivo* physiological functions and development of IELs on the front line of the intestinal mucosa. Research over the past 30 years has shown that IELs in mice and humans, especially those in the small intestine of mice, are a phenotypically and functionally distinctive subpopulation of peripheral T cells that is distinguished from so-called proper T cells, which are distributed in peripheral lymphoid tissues such as the spleen and lymph nodes (LNs) after development in the thymus (10). A vast majority of T cells found in peripheral lymphoid tissues of mice and humans are αβ T cells, while only a few γδ T cells are present. In contrast, IELs in mice and humans include large numbers of cells expressing αβTCRs and those expressing γδTCRs. From a study of IELs in athymic (nu/nu) mice, it is clear that many γδ-IELs are present, although the population size is decreased. In addition, in spite of the sharp decrease in αβ-IELs, meaningful numbers of these cells can be detected. Thus, a substantial proportion of γδ-IELs seems to be generated and/or expanded in the absence of the thymus. In contrast, it is well known that both γδ T cells and αβ T cells are virtually undetectable in the spleen and LNs of nu/nu mice (11, 12). Functional aspects of IELs have been adequately explained in other reviews in this volume, and this review contains personal insights concerning the past, present, and future of extrathymic development of IELs and where the research is heading. Furthermore, we discuss how IELs settle down in the IEC layer through Bm and emphasize how they behave and survive in the IEC layer in situ.

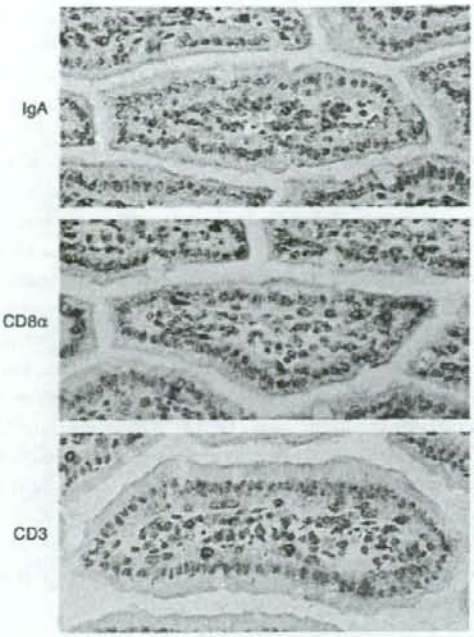

Fig. 1. Immunohistochemical visualization of IgA-, CD8α-, and CD3-expressing cells in jejunal villi. Note that IgA^+ B cells are localized exclusively in the LP, whereas that $CD8\alpha^+$ T cells, namely intestinal IELs, are compartmentalized above the Bm in the IEC layer of the small intestine. In contrast, in addition to numerous IELs in the IEC layer, $CD3^+$ T cells, mostly $CD4^+$ T cells, are also found in the LP of the villi.

IEL development in the intestinal epithelium: evolutionary perspective

The intestine was the first organ to appear when animals became multicellular; even though some multicellular animals lacked brains, there were none without intestines. To defend the intestines against pathogenic microorganisms and harmful substances from the exterior, macrophage-like lymphoid cells developed directly under the intestinal epithelium. The first organ to appear in our living body is the primordial gut, and many organs, including lungs, liver, pancreas, and thyroid gland, are derived from this apparatus. Some marine animals breathe through gills that develop from the upper digestive tract, and pulmonary respiration evolved with the change from marine to terrestrial life. It is well known that the thymus was derived from part of the gill. Therefore, all these organs have

a kindred relation, and latent production of lymphocytes appears possible. In agreement with this argument, fetal liver of mammals including mice is a primary lymphoid organ producing lymphomyeloid cells.

The IEC layer was proposed as a lymphocyte-producing organ as early as 1967 (13). Gut-associated lymphoid tissue (GALT), which contains about 60% of all peripheral lymphocytes, monitors and defends the intestinal mucosa in most vertebrates. T cells and antibodies, the key players in adaptive immunity, have not been found in the jawless fish *Agnatha*, the oldest phylogenic vertebrate lacking a thymus, spleen, and LNs (14, 15). However, GALT, characterized by many lymphoid cells, is found in *Agnatha* such as lampreys and hagfish, and the intestinal mucosa of such animals appears to serve as lymphocyte production sites (14, 15). Furthermore, the bursa of Fabricius, a GALT of birds, and Peyer's patches (PPs) of ruminants are also well known as primary lymphoid tissues responsible for the development of B cells (16). If we consider the large amount of knowledge based on animal evolution, there is nothing remarkable about development of IELs in intestinal mucosa in situ in mice and humans.

Findings supporting extrathymic development of murine IELs

Type a and type b IELs

IELs of the murine gut have been identified as ill-defined T cells that lurk in the anatomical front of the intestine (1, 3–9), with a primarily cytotoxic T-cell phenotype (4, 17–19). A large fraction of murine IELs bearing CD8αα homodimers ($CD8\alpha\alpha^+$ IELs) have been proposed to originate locally through a differentiation process initiated in $c\text{-}kit^+IL\text{-}7R^+$lineage marker $(Lin)^-$ gut precursors. Hayday *et al.* (10) have proposed that the functional complexity and phenotype heterogeneity of IELs might be simplified if IELs are classified into just two cell types: 'a' and 'b'. Type a includes $CD4^+$ and $CD8\alpha\beta^+$ αβ-IELs that primarily recognize antigens presented by classical major histocompatibility complex (MHC) class I and class II molecules and are primed within the systemic circulation. Type b IELs include $CD8\alpha\alpha^+$ αβ- and γδ-IELs that respond to antigens not restricted by classical MHC molecules. Although $CD8\alpha\alpha^+$ αβ- and γδ-IELs are clearly different from one another, type b IELs share many 'unconventional' features that distinguish them from type a IELs.

Although dependence of the type a $CD8\alpha\beta^+$ and type b $CD8\alpha\alpha^+$ αβ-IELs but not type b $CD8\alpha\alpha^+$ γδ-IELs on MHC class I molecules was reported using β2-microglobulin (β2m)-deficient mice (20, 21), a recent analysis of gene expression profiles between type b $CD8\alpha\alpha^+$ αβ− and γδ-IELs showed a high degree of similarity (22). These two classes of IELs are not only related functionally but also have a kindred relation.

The total number of αβ-IELs decreased sharply in $\beta2m^{-/-}$ mice due to the disappearance of both $CD8\alpha\beta^+$ (type a) and $CD8\alpha\alpha^+$ (type b) subsets. In β2m/TCR-δ double-mutant mice, which lack β2m and γδ-IELs, the $CD8\alpha\alpha^+$ subset expanded dramatically, while the $CD8\alpha\beta^+$ subset did not (Fig. 2). Thus, in the absence of γδ-IELs, αβ-IELs in β2m-deficient mice outnumbered those in wildtype littermates due to considerable expansion of type b $CD8\alpha\alpha^+$ αβ-IELs (Fig. 2). These results (23) indicate that generation of type b $CD8\alpha\alpha^+$ αβ- and γδ-IELs is essentially β2m independent, while generation of type a $CD8\alpha\beta^+$ αβ-IELs is highly dependent on β2m-MHC class I

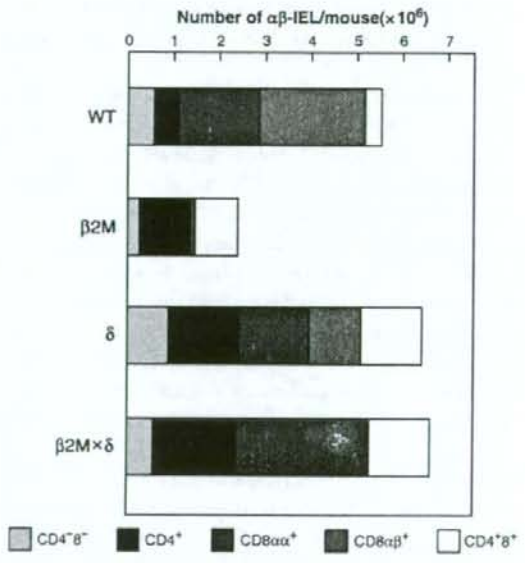

Fig. 2. Composition of αβ-IEL subsets in wildtype (WT), β2m-deficient, TCR-δ mutant (δ), and β2m × TCR-δ double-mutant (β2m × δ) mice. These four different mice were littermates of the F_2 generation of an intercross between $\beta2m^{-/-}$ and $\delta^{-/-}$ mice. IELs isolated from these mutant mice were incubated first with anti-CD8α monoclonal antibody (biotinylated) and then with streptavidin–allophycocyanin. After washing, the IELs were counterstained with two combinations of two phycoerythrin-conjugated monoclonal antibodies (anti-CD4 and anti-CD8β) and two fluorescein-isothiocyanate-conjugated monoclonal antibodies (anti-αβ TCR and anti-γδ TCR, respectively). Absolute numbers of double-negative ($CD4^-CD8^-$), single positive ($CD4^+$, $CD8\alpha\alpha^+$, or $CD8\alpha\beta^+$), and double positive ($CD4^+CD8^+$) subsets in the αβ-IEL population were calculated on the basis of total number of αβ-IELs. Note that $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ αβ-IEL subsets are absent from the small intestine of β2m mutant mice, whereas the $CD8\alpha\alpha^+$ but not $CD8\alpha\beta^+$ αβ-IEL subset expands markedly in the small intestine of double-mutant β2m × δ mice, namely β2m mutant mice that lack γδ-IELs.

molecules expressed by the controlling cells at the type a IEL precursor development site. These findings suggest the possibility that type b IELs, CD8αα$^+$ αβ- and γδ-IELs, develop in the same anatomical site(s). When no β2m-MHC class I molecules are present, development of CD8αα$^+$ αβ-IELs is likely inhibited because development of CD8αα$^+$ γδ-IELs surpasses that of CD8αα$^+$ αβ-IELs competitively.

CD8αα exerts a specific and high affinity for interaction with the non-classical MHC class I molecule thymus leukemia (TL) antigen, which is expressed abundantly by murine thymic stromal cells and by IECs (24). It was also proposed that CD8αα$^+$ TCR-αβ T cells originated from the thymus through agonist-dependent positive selection (25). The mechanism of development of CD8αα$^+$ T cells and *in vivo* physiological functions, including whether or not this scenario is correct, remain to be clarified.

Evidence obtained in a study of athymic *nu/nu* mice

The evidence that most clearly supports thymus-independent development of gut-oriented type b IELs is obtained from a study of T cells in the athymic (nu/nu) mouse. Almost no γδ T cells or αβ T cells are observed in the spleen and LNs of nu/nu mice. A considerable population of γδ-IELs is present in IELs of nu/nu mice, and αβ-IELs can also be detected (Fig. 3). Since these αβ-IELs are CD8αα$^+$ type b IELs and no αβ-IELs are found in TCRβ$^{-/-}$ mice (Fig. 3), it is evident that a few type b αβ-IELs develop independently of the thymus.

Many reports have been published on the thymus-independent development of type b IELs. These findings include the presence of a few CD3$^-$ lymphocytes in the IELs and the resulting possession of precursor T-cell-like properties, i.e. the fact that these tentative precursors observed in mice and humans retain messenger RNA (mRNA) for recombination-activating gene-1 (RAG-1) and RAG-2 and pre-Tα molecules. For details, the reader is referred to previously published articles (8, 9, 26–30).

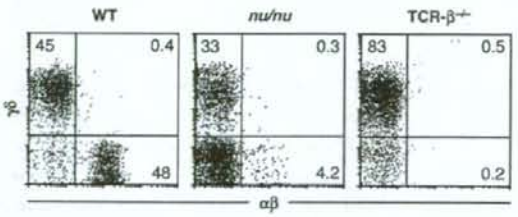

Fig. 3. Composition of αβ- and γδ-IELs isolated from wildtype (WT), athymic (nu/nu) and TCR-β$^{-/-}$ mice. Flow cytometric analysis of IELs isolated from five individuals each of three different strains of mice was performed, and the representative profiles of IELs are presented. In this case, absolute numbers of IELs recovered were 5.4×10^6 from WT mice, 2.3×10^6 from nu/nu mice, and 5.3×10^6 from TCR-β$^{-/-}$ mice. The percentage of αβ- and γδ-IELs in the corresponding quadrants is shown. Note that αβ-IELs are drastically reduced in the athymic condition. Nevertheless, a meaningful number of αβ-IELs are still present in the small intestine of the nu/nu mouse compared with the total absence of αβ-IELs from the TCR-β$^{-/-}$ mouse. In contrast, there are few, if any (<1%), αβ T cells in the spleen and MLNs of this same nu/nu mouse (data not shown).

Here, we introduce our results (31) from screening lymphoid tissues of athymic (nu/nu) RAG-1$^{GFP/+}$ animals, which have the green fluorescence protein (GFP) gene in the RAG-1 locus. Only CD19$^+$ B cells (32) in the bone marrow (BM), spleen, mesenteric lymph nodes (MLNs), and PPs express RAG-1, while in IELs, a meaningful number of CD19$^-$ cells express RAG-1, although the amount of RAG-1 molecules expressed is low (Fig. 4). Since CD19$^-$RAG-1^{low} IELs are CD3$^-$ and are not observed in IELs of RAG-1$^{+/+}$ mice, this finding supports the distribution of small numbers of RAG-1^{low} precursor T cells in IELs. DETCs of wildtype mice that express homogenous γδTCRs (Vγ5Jγ4Cγ1 and Vδ1Jδ2Cδ) are known to be derived from the first wave of fetal γδ thymocytes, which are produced in the thymus at about antenatal day 15 (2). Therefore, even though Vγ5$^+$ DETCs are not present naturally, it has been shown that Vγ1/Vδ6$^+$ DETCs are present in the epidermis of nu/nu mice (33). These findings together with those reported by Matis et al. (34) and Yoshikai et al. (35) prove that the thymus-dependent type a subset (2) and the thymus-independent type b subset (33–35) such as γδ T cells are present in peripheral anatomical sites other than the IEC layer.

A new member of GALT: cryptopatches

Discovery of cryptopatches in mouse small intestine as the precursor IEL-producing site

We have shown that multiple tiny lymphoid cell aggregations, filled with about 1000 closely packed c-kit$^+$IL-7R$^+$Thy1$^+$ CD3$^-$B220$^-$ lymphocytes, colonize throughout the small intestinal mucosa of C57BL/6 mice (36). The location is in the crypt LP [cryptopatches (CPs)]. They are first detected in the third week of postnatal life in C57BL/6 mice. In terms of morphogenesis, cellular composition, and fine tissue structure, neither PPs nor isolated lymphoid follicles (ILFs) are identical to CPs (30, 37). CPs contain neither cells undergoing apoptosis nor cells bearing RAG-1 molecules but do contain dendritic stromal cells bearing CD11c/CD18 molecules. The presence of transcripts for germ line TCR genes and mRNA for proteins involved in TCR rearrangement (38) and the ability of c-kit$^+$Lin$^-$ CP cells to generate TCR$^+$ IELs in T-cell-deficient mice (39) indicate that at least some CP cells are committed to the T-cell lineage and are competent for generation of

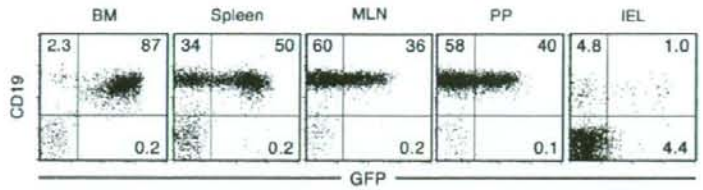

Fig. 4. A small number of RAG-1-expressing lymphocytes are present in the intestinal intraepithelial compartment of nu/nu mice. Lymphocytes from BM, spleen, MLNs, PPs, and IEC layer (IELs) were isolated from nu/nu mice carrying a *GFP* gene in place of the *RAG-1* gene (nu/nu RAG-1$^{GFP/+}$ mice) (31) and then were subjected to flow cytometric analysis. Note that only in the IEL preparation, a meaningful number of GFP-dull-positive cells are present. Importantly, these cells are not B-lineage cells because they are CD19 negative.

thymus-independent type b IELs, especially CD8αα$^+$ γδ-IELs (12).

To obtain direct evidence for generation of type b IELs from the precursors that settle in CPs, cytokine receptor γ chain mutant nu/nu mice that lack a thymus, PPs, CPs, and intestinal T cells (40) were reconstituted with wildtype Ly5.1$^+$ BM cells. BM-derived TCR$^-$ IELs first appeared within villous epithelia of small intestine overlaying regenerated CPs, and these TCR$^-$ IELs subsequently emerged throughout the epithelia. Thereafter, TCR$^+$ IELs increased sluggishly to a number comparable with that in athymic nu/nu mice and consisted of both αβ- and γδ-IELs (38). Taking all these results together, CPs are the first murine GALT to be identified that most likely serve as the site of development of lymphohemopoietic precursors for type b IEL descendents at commencement of weaning (36, 38, 40). Recent data showed a clonal relationship between CP T cells and γδ-IELs (41).

Evidence that CPs are not the precursor IEL-producing site

By characterizing phenotypically distinct lineage-negative populations in the CPs and gut epithelium, Lambolez *et al.* (42) showed that only 3% of CP cells were clearly involved in T-cell differentiation and suggested that these CP structures may have an additional physiological role in the gut. In contrast, Guy-Grand *et al.* (43) clarified the following from an examination of RAG-2 expression using GFP transgenic mice evaluated by GFP (carrying a *GFP* reporter gene driven by the RAG-2 promoter). In nu/nu mice, T lymphopoiesis occurs mainly in MLNs, less in PPs, and not in CPs. Importantly, this extrathymic T lymphopoiesis is totally repressed in euthymic mice (43). Based on these findings, Guy-Grand *et al.* (43) concluded that in normal euthymic mice, all gut αβ-IELs, including type b CD8αα$^+$ αβ-IELs, are of thymic origin. It has also been shown that thymus transplantation into nu/nu mice results in the appearance of thymus graft-derived αβ- and γδ-IELs in nu/nu recipients (44). To evaluate this important issue, we generated nu/nu aly/aly (*alymphoplasia*) double-mutant mice lacking thymus, all LNs, PPs, and ILFs but possessing CPs (12). Substantial colonization by γδ-IELs comprising the major CD8αα$^+$ subset took place, and use of TCR-γ-chain variable gene segments by these γδ-IELs was unaltered (12). These findings indicate that MLNs and PPs are not an absolute requirement for development of γδ-IELs but instead support the notion that gut CPs generate progenitor γδ-IELs, even under athymic conditions. However, absolute numbers of γδ-IELs from nu/nu aly/aly mice are smaller than those from the corresponding control nu/nu mice (12). These features indicate that LNs and PPs in fact determine the number of γδ-IELs under the nu/nu conditions. In any event, there would be a hierarchy of T-cell production in terms of anatomical sites, although the detailed mechanism is still not well understood. By a mechanism such as the tropic effect of thyrotropin-releasing hormone on IEL development (45, 46), however, almost all αβ-IELs appear to originate in the thymus in euthymic mice. In nu/nu mice, however, IELs appear to be generated mainly by the MLNs and PPs, and in nu/nu aly/aly mice that lack a thymus, all LNs, PPs, ILFs, and αβ- and γδ-IELs appear to be generated from other anatomical sites, probably CPs. In this context, it has been reported that T-cell-committed precursors are distributed not only in the thymus but also in the BM, liver, and spleen (47–49). It is inferred based on these findings that extrathymic T-cell generation has not been proven to be repressed completely in normal euthymic mice. In other words, there is no solid evidence denying a possibility that extrathymic T cells are generated in euthymic conditions.

It has recently been shown that retinoic acid-related orphan receptors (RORγt) detected in fetal lymphoid tissue-inducer (LTi) cells are also expressed in cells within gut CPs and that, by fate mapping of RORγt$^+$ cells, type b IELs, such as γδ-IELs, are not the progeny of RORγt$^+$ CP cells (50). However, it remains an open question whether a small fraction of lymphoid cells in CPs does not express RORγt or all CP cells express RORγt. To investigate this point in detail, we generated nu/+ RORγt$^{GFP/+}$

mice and nu/nu RORγt$^{GFP/+}$ mice and obtained the results shown in Fig. 5.

In agreement with the report of Eberl and Littman (50), many RORγt^{+} (GFP^{+}) cells were present in thymocytes from nu/+ RORγt$^{GFP/+}$ mice, although GFP expression was weak (unpublished observation). Analysis of CP cells from nu/nu RORγt$^{GFP/+}$ mice showed that almost all GFP^{+} cells were interleukin-7 receptor positive (IL-7R)$^{+}$ and CD8α^{-}. However, CD3^{-} cell subsets showing various phenotypes such as IL-7R^{+}GFP^{-}, c-kit^{+}GFP^{+}, c-kit^{+}GFP^{-}, c-kit^{-}GFP^{+}, Thy-1^{+}GFP^{+}, Thy-1^{+}GFP^{-}, Thy-1^{-}GFP^{+}, CD4^{+}GFP^{+}, CD4^{+}GFP^{-}, CD4^{-}GFP^{+}, CD8^{+}GFP^{-}, Lin^{+}GFP^{+} (majority of them CD4^{+}GFP^{+}), Lin^{+}GFP^{-}, and Lin^{-}GFP^{+} were all present but with variable population sizes (Fig. 5A). In addition to the colonization of γδ-IELs and small numbers of αβ-IELs, of particular note was the presence of CD3^{-} IELs showing c-kit^{+}GFP^{+} and IL-7R^{+}GFP^{+} phenotypes in IEC compartments of nu/nu RORγt$^{GFP/+}$ mice (Fig. 5B). These new findings do not necessarily support the conclusion of Eberl and Littman (50) and show distribution of RORγt^{-} c-kit^{+}, IL-7R^{+}, Thy-1^{+}, CD4^{+}, CD8^{+}, and/or Lin^{+} lymphocytes in murine gut CPs. In this context, we previously reported (36) that CPs are not detectable in IL-7R$^{-/-}$ mice. However, although γδ-IELs are absent owing to selective blockage of TCR-γ gene rearrangements (51), we noticed only a slight decrease in development of type b αβ-IEL subsets in IL-7R$^{-/-}$ mice. With these observations in mind, we reinvestigated hundreds of cryosections prepared from small intestines of IL-7R$^{-/-}$ mice by immunohistochemistry and verified that conspicuously emaciated CPs filled with c-kit^{+} cells and decreased by more than 16-fold in number were present in this mutant intestine (40). Similarly, although mice genetically deficient in lymphotoxin α (LTα) have been reported to lack CPs (52), we observed that histogenesis of CPs and intestinal development of αβ- and γδ-IELs remained almost intact in LTα$^{-/-}$ mice (37). In consideration of our research results, i.e. CPs are observed not only in IL-7R$^{-/-}$ mice but also in LTα$^{-/-}$ mice, the conclusion that CP development is not found in RORγt$^{GFP/GFP}$ mice lacking RORγt (50) seems to require careful reexamination. It is easy to ascertain the presence of CPs, but it is quite difficult to conclude that CPs are totally absent. At the same time, we should examine if type b IELs, such as γδ-IELs, develop from the RORγt^{-} c-kit^{+}GFP^{-} or IL-7R^{+}GFP^{-} subset (Fig. 5A) distributed in the CPs of nu/nu RORγt$^{GFP/+}$ mice.

What are these CPs?

Based on the results obtained using RORγt$^{GFP/+}$ mice, Eberl and Littman (50) proposed that the principal function of murine c-kit^{+}Lin^{-} RORγt^{+} CP cells is to induce formation of lymphoid

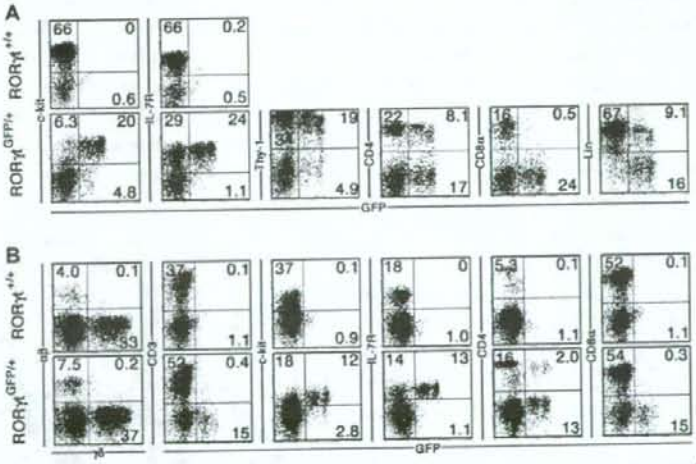

Fig. 5. Flow cytometric analysis of CP cells and IELs isolated from nu/nu mice carrying the wildtype RORγt genes (RORγt$^{+/+}$ mice) and carrying a GFP gene in place of RORγt gene (RORγt$^{GFP/+}$ mice). CP cells were isolated according to the method described previously (37). Although CP cells (A) and IELs (B) isolated from nu/nu RORγt$^{+/+}$ mice do not contain GFP^{+} cells, those isolated from nu/nu RORγt$^{GFP/+}$ mice contain a substantial population of GFP^{+} RORγt-expressing cells. Furthermore, these RORγt-expressing cells from CP and IEL compartments of nu/nu RORγt$^{GFP/+}$ mice appear to be composed of two discrete RORγt^{high} and RORγt^{low} cell subsets. 'Lin' in A represents lineage markers CD3, B220, Mac-1, Gr-1, TER119, CD11c, CD4, and CD8α. Lymphocytes isolated from spleen, MLNs, and PPs of nu/nu RORγt$^{GFP/+}$ mice lack GFP^{+} RORγt-expressing cells (data not shown).

follicles, namely ILFs, in the LP in a manner similar to induction of LNs and PPs by RORγt^+ Lti cells. It is also possible that CP cells are precursor cells of gut-oriented lymphomyeloid cells other than IELs (42). The CPs of mice have been discounted as the anatomical site where precursor IELs congregate, as no CP-like lymphoid clusters have been reported in the intestinal mucosa of mammals other than mice. However, since evidence indicates development of gut-oriented T cells, mainly IELs, in the intestinal mucosa of humans (53–59) and rats (60), to determine whether or not CPs and CP-like lymphoid tissues are present in enteric mucosa of animals is an important goal for future experiments. We (61) showed that lymphocyte clusters, just like the structure named lymphocyte-filled villi (LFV) (62), populated predominantly with c-kit^+IL-$7R^+$ cells and less with $\alpha\beta$TCR cells, were found distributed throughout the length of the small intestine of rats. Nevertheless, we were unable to verify whether these rat LFV, containing undifferentiated lymphocytes, represented clusters of extrathymic precursor T cells. With regard to this same issue, several groups have actively sought evidence of CPs in the human gastrointestinal tract, illuminating distinctive T-cell facets of human fetal gut lymphocytes (58, 59). In agreement with these findings, we have identified multiple tiny c-kit^+ lymphoid cell clusters in human fetal intestine at the second trimester of fetal life (unpublished observation). Overall, we take it for granted that the differentiation of type b IELs is not exactly the same among different vertebrates. For instance, if epidermal immune regulation by $\gamma\delta$-DETCs in mice is of considerable physiological importance, how do other mammals including humans cope without $\gamma\delta$-DETCs? In these animals, immunoregulatory function should be provided by some lymphoid cells other than $\gamma\delta$-DETCs.

Based on these findings, precursors of type b IELs that develop extrathymically in humans and rats appear to be produced in CP-like lymphoid tissues of mice during fetal life. Then, the precursors are dispersed throughout the LP or IEC layer, or self-renewal is localized in the IEC layer after expression of the $\alpha\beta$TCR or $\gamma\delta$TCR and completion of development, as in the case of B-1 B cells. Mouse DETCs (2) expressing homogenous Vγ5/V$\delta1^+$ $\gamma\delta$TCRs are produced only in fetal thymus in a very limited period at about day 15 of fetal life. No such Vγ5/V$\delta1^+$ $\gamma\delta$-DETCs are found in the epidermis of athymic nu/nu mice (33), while homogenous Vγ5/V$\delta1^+$ $\gamma\delta$-DETCs of euthymic mice are present throughout life (1.5–2 years). If the supply of Vγ5/V$\delta1^+$ $\gamma\delta$-DETCs occurs only in the first wave of fetal $\gamma\delta$ thymocytes development, the life of Vγ5/V$\delta1^+$ $\gamma\delta$-DETCs must be as long as the life of the mouse. Is this really the case? We do not think that it is. It is possible that precursor Vγ5/V$\delta1^-$ $\gamma\delta$-DETCs generated by fetal thymus at about day 15 of fetal life lurk somewhere in the body (such as in the epidermis or dermis), develop at a fixed pace, and produce new Vγ5/V$\delta1^+$ $\gamma\delta$-DETCs. It is also possible that $\gamma\delta$-DETCs expressing homogenous Vγ5/Vδ1-TCRs continuously undergo self-renewal in the epidermis. Indeed, $\gamma\delta$-DETCs are shown to recognize and respond to antigens expressed on damaged, stressed, or transformed keratinocytes by means of their TCRs and produce keratinocyte growth factors (63), and it has been indicated that continuous stimulation of Vγ5/Vδ1-TCR^+ $\gamma\delta$-DETCs by the relevant ligand(s) is critical for maintenance of $\gamma\delta$-DETCs throughout the life of animals (64). In this context, it is conceivable that extrathymically generated mature human and rat IELs, which originate from cells settling in CP-like lymphoid tissues of mice during fetal life, undergo continuous self-renewal over the entire postnatal life of these animals by stimulation with relevant gut-associated antigens. In conclusion, although much remains to be learned about the mysterious development of extrathymic T cells in general and also the enigmatic features of CPs before we evaluate them as anatomical sites in which murine type b precursor IELs develop, many aspects of extrathymic T-cell immunobiology are now coming together step-by-step.

Where do these IELs come from?

Many types of cells are present outside the Bm of the small intestine (Fig. 6). Cells other than IELs present in the IEC layer are known to develop from stem cells located in the crypt. As described in the Introduction, IELs must migrate into the IEC layer from LP across the Bm. Immunohistochemical examination has shown that there are at least 2, or many more, IELs for every 10 IECs in mouse small intestine (65, 66). Thus, absolute numbers

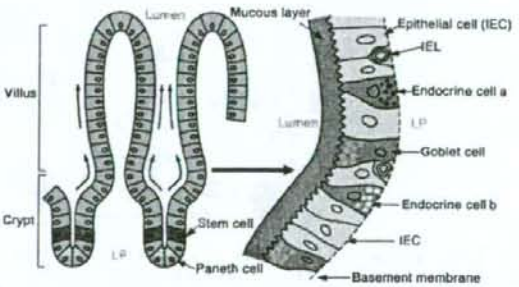

Fig. 6. Schematic illustration of cells that settle in the IEC layer of the small intestine. Every cell that settles in the IEC compartment, except IELs on the right of this figure (magnified), is the progeny of the stem cells shown on the left of this figure. Endocrine cells a and b contain different gut hormones.

of murine IELs are estimated to account for about half of the peripheral T-cell pool (67). However, it is not known if this huge IEL population enters the IEC layer across the Bm at sites such as crypts or villi, as shown in Fig. 6, or if IELs that have entered the IEC layer later cross the Bm and return to the LP. A large fraction of IELs is composed of γδ-IELs, and the fact that γδ T cells are almost absent from the LP compartment of villi in normal mice indicates that once they have entered the IEC layers, IELs and/or precursor IELs might return to LP in rare cases. By electron microscopy, we (39) showed that numerous lymphocytes cross the Bm that comes into contact with the CPs. Furthermore, by analysis of BM-chimeric mice, we (38) verified that donor BM-cell-derived IELs first appeared within the IEC layer of villi around regenerated CPs filled with BM-cell-derived c-kit$^+$ cells. These experimental results, in conjunction with a wide range of evidence showing the presence of small numbers of CD3$^-$ precursor IEL-like lymphocytes in the IEC compartment (8, 9, 26–30, 68), suggest a scenario in which precursor IELs that developed in CPs enter the IEC layer from the Bm that overlays CPs, and then these cells very sluggishly develop into mature type b IELs. In contrast, no definite findings have been obtained on whether type a αβ-IELs, such as CD4$^+$ or CD8αβ$^+$ T cells derived from the thymus, cross the Bm by some route and enter the IEC layer.

Parabiotic C57BL/6 Ly5.1 and Ly5.2 mice sharing circulation have been used by several groups (69, 70) to examine whether c-kit$^+$ stem cells that settle in local organs give rise to lymphoid cells in these organs in situ. As anticipated, the partner cells mixed rapidly in the spleen, all LNs, and PPs. In contrast, there were no or very few mixtures of partner cells in the thymus, CPs, and IELs (70). Poussier et al. (69) reported that IELs in the murine intestine did not mix together in parabiotic Ly5.1 and Ly5.2 mice. Overall, these findings appear to once again support the notion that IELs arise from their own pre-existing local precursor cells and that neighboring CPs continuously supply these local precursor cells at unknown rates.

What are these IELs?

There are still many riddles concerning the behavior and biological function of IELs that settle in the anatomical front of the IEC compartment in situ. Some of these unresolved issues are discussed below. We also discuss the development of research yet to be undertaken and future perspectives.

Behavior

Epithelial stem cells (Fig. 6) proliferate at the base of the crypts. Newly formed cells move upward and differentiate into various types of cells, mostly IECs (Fig. 6), in a process called migration-associated differentiation. The entire process of migration toward the top of the villi takes only several days, and the cells die there, most likely by apoptosis (71).

Electron microscopic examination of immersion-fixed tissue sections shows that IEL interdigitate tightly with IEC at the basolateral faces of IEC (Fig. 7A). Most IELs are terminally differentiated cells or those in the G0/G1 phase of the cell cycle.

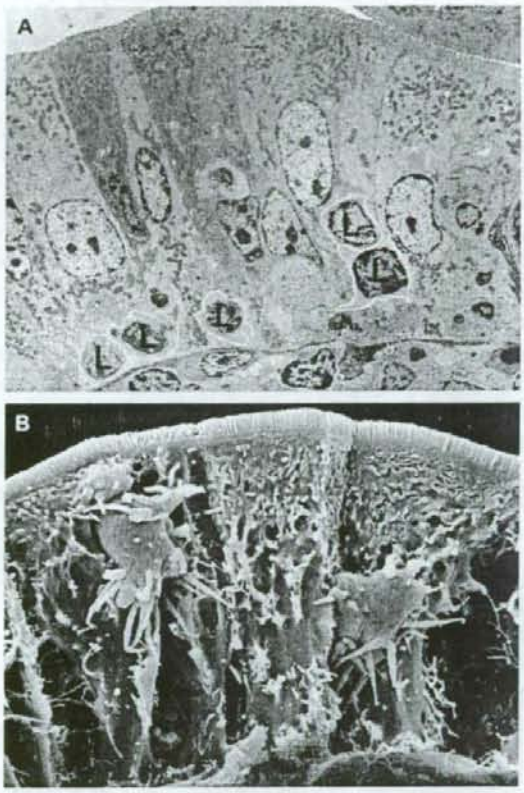

Fig. 7. Electron microscopic analysis of small intestinal epithelium. (A) Transmission electron micrograph showing IELs in intestinal epithelium obtained from an immersion-fixed sample. At least five IELs (L, edged with yellow line) are encased within the epithelium. No free space is visible among epithelial cells in the immersion-fixed samples; IELs appear to be tightly packed in the epithelium. (B) Scanning electron micrograph of IELs in a perfusion-fixed material. Two lymphocytes (yellow) equipped with spine-like processes are detected among epithelial cells. Note the broad free space at the basal region of the epithelium. A perfusion-fixed sample retains more precise and physiological three-dimensional structure of the tissues than that obtained by an immersion-fixed sample because the tissues shrink totally during and after the immersion-fixation. [This image is a modified reproduction of our figure that appeared in Cell & Tissue Research, used with kind permission of Springer Science and Business Media (83)].

It has been shown by bromodeoxyuridine labeling experiments that the average half-life of murine IELs is 3 weeks or much longer (72). If we assume that IELs are also migrating to the top of the villi, IECs must continuously get ahead of IELs, suggesting that individual IELs are capable of coming in contact with and/or surveying a significant number of IECs. Are IELs really migrating to the top of the villi? If so, at what speed are they migrating? At present, we do not know whether IELs can actually move upward or whether they maintain a rather stationary position.

Fig. 7B shows a vertically fractured face of perfusion-fixed small intestine by scanning electron microscopy. As is clear from this image, the surface in contact with intestinal lumen (apical surface of IEC) forms a tight junction between the IECs and is covered with microvilli with no gaps. However, there are relatively wide gaps between cells just on the Bm, and IELs are not always fixed by tight interdigitation with the lateral faces of the IECs. They appear to move to and fro relatively freely. IELs are also often in contact with IECs through process-like structures (Fig. 7B). In this image, it looks like the IELs are completely covered with trees, as if it were a mighty jungle viewed from the air, but there are considerable gaps near the ground under the trees where lions (≈ IELs) sleep and animals such as leopards (≈ IELs) lurk and move around (Fig. 8). In any case, clarification of the life and behavior of these mature IELs and the small number of precursor IELs in the IEC compartment are important topics for future study.

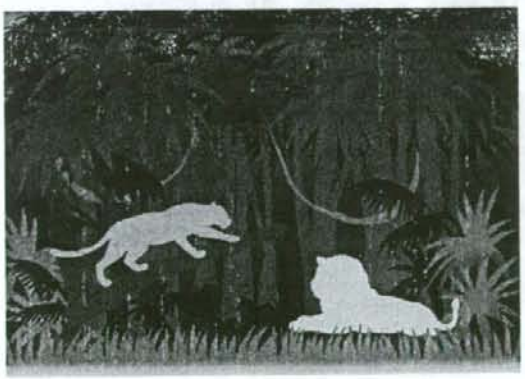

Fig. 8. A pictorial representation of the mighty jungle of the IEC layer. Based on the three-dimensional scanning electron microscopic picture of the IEC layer presented in Fig. 7B, we illustrated a mighty jungle in which a lion (≈IEL, with potentially cytotoxic function?) is resting on the ground (Bm) and a leopard (≈ IEL, like an indiscriminate predator?) is moving around a tree trunk (lateral face of IEC). It is evident that there is considerable room between the tightly packed treetop and the surface of the ground where these animals (≈ IELs) might be freely moving to and fro.

Oligoclonality

Whereas IELs are potentially able to use multiple Vβ TCR genes, both human (73–75) and murine (76) αβ-IELs are known to be derived from a limited number of oligoclonal T-cell clones. This oligoclonality of IELs points to the presence of a restricted set of foreign and/or self-antigens in the gut that may be ligands involved in the stimulation and expansion of these gut T cells. The large numbers of T cells distributed in the spleen and LNs appear to be inexperienced or virgin T cells that have not received antigen stimulation in normal specific pathogen-free mice. In contrast, T cells in the gut of specific pathogen-free mice are activated cells, continuously receiving antigen stimulation. As a result of selection, only competent clones expand and become dominant. Under these circumstances, even though the types of antigens are very diverse and are not limited to a restricted set of foreign and/or self-antigens, the TCR of αβ-IELs eventually results in oligoclonality. One mysterious and unexpected finding is that genetically identical individuals, even from the same litter and housed in the same cage, show distinct and apparently non-overlapping oligoclonal repertoires of both type a and type b αβ-IELs (76). Is the immune response to a diverse range of external antigens in the intestinal lumen really involved in the establishment of the oligoclonality of αβ-IELs? There is no firm experimental evidence to answer this question. In this respect, the finding that αβ-IELs in germ-free mice are also oligoclonal (77) is very intriguing and shows that αβ-IELs are oligoclonal, even when huge numbers of intestinal flora-derived antigens and microbe-associated immunoactive substances are not present.

Cytotoxicity

IELs are mostly terminally differentiated and activated T cells that possess a granular cytoplasmic structure containing perforin and granzyme (78), capable of killing Fc-receptor-bearing target cells after bridging them with anti-CD3, anti-αβTCR, or anti-γδTCR monoclonal antibodies (78–80). However, at most 10^7 IELs per mouse from the small intestine can be isolated for *in vitro* analysis, despite the fact that $5–10 \times 10^7$ IELs have been found to settle in the IEC compartment by immunohistochemistry (67) (Fig. 1). Therefore, we might have studied *in vitro* only a portion of the IELs, which can be isolated easily from intestinal mucosa as a subpopulation maintaining their typical granular cytoplasmic structure and cytotoxic activity. In this context, of great importance in the future is to examine *in vitro* characteristics of the large number of IELs that cannot be recovered using current techniques and/or are lost in the process of purification in a test tube. Such a 'missing' population should be examined,

if the cells maintain their characteristics of terminal differentiation. γδ-IELs from germ-free mice are known to show cytotoxicity (81) in the same way as αβ-IELs in germ-free mice show oligoclonality. Electron and light microscopy shows that murine IELs from scid/scid (severe combined immunodeficient) mice that are unable to generate αβ- and γδ-IELs have granulated IELs similar to those in normal mice (82), are $CD3^-CD8\alpha\alpha^+$ IELs are present in scid/scid mice and nu/nu scid/scid mice (40). In addition, findings showing that $CD3^-CD8\alpha\alpha^+$ IELs are not present in cytokine receptor γ chain mutant nu/nu mice lacking CPs (38, 40) suggest that a special but as yet unknown microenvironment in CP and IEC compartments could dictate the ability of precursor IELs entering the IEC layer to possess a granular cytoplasmic structure and to express CD8αα molecules with no relation to presence or absence of a thymus, TCR expression, or intestinal flora.

It is well known that antigen-specific αβ-IELs have a protective role against infection by pathogenic microorganisms, but ligands of cytotoxicity expressed by αβ-IELs and γδ-IELs from specific pathogen-free mice and γδ-IELs from germ-free mice are still not clear. Do these cytotoxic IELs present in the harsh microenvironments at the front line of intestinal mucosa correctly identify target cells by TCR? Even without very strict specificity, are IECs that have been damaged by stress, viral infections, bacterial infections, or transformed IECs rapidly detected and are these dubious IECs eliminated? Are these cytotoxic IELs indiscriminate predators lurking savagely in the jungle of the IEC layer (*Fig. 8*)? What T cells are these IELs anyway? In any event, elucidation of these conditions is very important to clarify the development and physiological function of IELs.

Conclusion

IELs are known as peripheral T cells with marked specificity and a very large population size, but their development and physiological function remain a mystery. Findings obtained through research over the past 30 years are very important. We view these diverse experimental observations as many tips of an iceberg that must firmly interconnect with its hidden part. Understanding the immunobiology of IELs must begin in the context of intestinal flora, which outnumber the total number of cells in our body and which have evolved with us in a commensal or symbiotic state. Elucidation of the characteristics of immune responses in the intestines, as the frontline defense against pathogens from the outer world, through clarification of the development and physiological function of IELs is essential for manipulation of intestinal immunity for our benefit.

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Subcutaneous Adipose Tissue–Derived Stem Cells Facilitate Colonic Mucosal Recovery from 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS)–Induced Colitis in Rats

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Background: Adipose tissue–derived stem cells (ADSCs) can be easily obtained from subcutaneous adipose tissue, and ADSCs can be demonstrated to display multilineage developmental plasticity. In this study, using TNBS-induced colitis rats, we show the feasibility of repairing injured intestinal mucosa with adipose tissue–derived stem cells.

Methods: The subcutaneous adipose tissue of F344 rats was obtained and digested by collagenase. The digested tissue was cultured in DMEM containing 10% FBS for 1 month. ADSCs were confirmed to differentiate under appropriate conditions into various lineages of cells, including bone, neural cells, adipocytes, and epithelial cells. HGF, VEGF, TGF-β, and adiponectin in the culture supernatants of ADSCs were determined by ELISA. ADSCs (10^7 cells) were injected into the submucosa of the colon to examine their capacity to repair intestinal mucosa injured by TNBS.

Results: In the experimental colitis model, the injection of ADSCs facilitated colonic mucosal repair and reduced the infiltration of inflammatory cells. High levels of HGF, VEGF, and adiponectin were detected in the culture supernatants of ADSCs. Moreover, injected ADSCs distributed to several layers of the colon, and some of them differentiated into mesodermal lineage cells.

Conclusions: ADSCs can accelerate the regeneration of injured regions in experimental colitis. HGF, VEGF, and adiponectin might be responsible for the regeneration of injured regions in the colon.

(Inflamm Bowel Dis 2008;14:826–838)

Key Words: HGF, VEGF, adiponectin, subcutaneous adipose tissue, Crohn's disease

Crohn's disease is characterized by chronic relapsing inflammation of the gastrointestinal tract. Evidence suggesting that various immune, genetic, and environmental factors influence both the initiation and progression of colitis has been accumulated. However, the precise mechanism or mechanisms underlying the development of this disease have yet to be clarified.

In recent years, several groups have reported the ubiquitous distribution of adult stem cells in various tissues and organs, including bone marrow, muscle, brain, skin, and, more recently, even in subcutaneous fat.[1] Among these adult stem cells, those in the subcutaneous fat, termed adipose tissue–derived stem cells (ADSCs), can be easily obtained with a relatively lower burden on donors; they can be easily harvested from subcutaneous adipose tissue by lipoaspiration. These ADSCs have been demonstrated to display multilineage developmental plasticity.[2] Furthermore, ADSCs have been reported to have less heterogeneity in their immunophenotype and multilineage differentiation ability[3,4] than do bone marrow–derived mesenchymal stem cells. Because of these advantages, clinical use of ADSCs for not only fat, bone, and myocardium reproduction but also spinal cord regeneration, vascularization, and the treatment of intractable ulcers has been carried out, and the development of further clinical applications is expected. Garcia-Olmo et al[5] reported in a phase I clinical trial of Crohn's disease that after an operation to close fistula, cell therapy using autologous ADSCs can facilitate fistula repair. However, ADSCs may have, in general, a potential to treat Crohn's disease and inflammatory bowel disease (IBD) in addition to the treatment for fistula

Received for publication August 10, 2007; accepted December 4, 2007.

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Supported by a grant from Haiteku Research Center of the Ministry of Education; a grant from the Millennium program of the Ministry of Education, Culture, Sports, Science and Technology; a grant from the Science Frontier program of the Ministry of Education, Culture, Sports, Science and Technology; a grant from the 21st Century Center of Excellence (COE) program of the Ministry of Education, Culture, Sports, Science and Technology; a grant from the Department of Transplantation for Regeneration Therapy (sponsored by Otsuka Pharmaceutical Company, Ltd.); a grant from Molecular Medical Science Institute, Otsuka Pharmaceutical Co., Ltd.; a grant from Japan Immunoresearch Laboratories Co., Ltd. (JIMRO); and by Grant-in-Aid for inflammatory bowel disease of the Ministry of Health, Labor and Welfare.

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DOI 10.1002/ibd.20382

Published online 5 February 2008 in Wiley InterScience (www.interscience.wiley.com).

observed in a subpopulation of patients with Crohn's disease. Therefore, the potential ability of ADSCs to improve mucosal healing of inflammatory lesions and the biological mechanism underlying the repair function of ADSCs should be examined. In the present study, using Crohn's disease models in rats in which the colonic mucosa is injured by TNBS, we attempted to treat the mucosal injury by a submucosal injection of ADSCs to facilitate mucosal recovery; we expected this to be a simple and safe procedure. It is noted that ADSCs injected intravenously might be trapped by the reticuloendothelial system in the liver or lung[6]; therefore, they could not be recruited to the intestinal mucosa. Thus, in this study, ADSCs were directly injected into the submucosa, and ADSCs actually facilitated the regeneration of the injured region (by TNBS injection). Furthermore, we also examined the potency of ADSCs to differentiate into various lineage cells and their production of growth factors such as HGF, VEGF, TGF-β, and adiponectin that may accelerate mucosal regeneration.

MATERIALS AND METHODS

Animals

F344/DuCrlCrlj rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan), and maintained for 1 to 2 weeks in our animal facilities before the start of TNBS treatment. Rats were maintained on a 12-hour light/12-hour dark cycle under pathogen-free conditions and had a standard diet and water until reaching the desired age (13 weeks).

Isolation and Culture of Rat Subcutaneous Adipose Tissue–Derived Stem Cells (ADSCs)

The subcutaneous adipose tissue of F344 rats was obtained by abdominal incision. The raw subcutaneous adipose tissue was washed extensively with sterile phosphate-buffered saline (PBS; Gibco, Invitrogen Corporation, Carlsbad, Calif.) to remove blood cells. The extracellular matrix was digested with a solution of type II collagenase (0.075%; Gibco, Invitrogen Corporation, Carlsbad, Calif.) in balanced salt solution (5 mg/mL; Gibco, Invitrogen Corporation, Carlsbad, Calif.) for 60 minutes at 37°C to release the cellular fraction.[5] Next, the collagenase was inactivated by the addition of an equal volume of RPMI (Sigma-Aldrich, St. Louis, Mo.) containing 10% fetal bovine serum (FBS; Invitrogen Corporation, Carlsbad, Calif.). The suspension of cells was centrifuged at 250g for 10 minutes. Cells were resuspended in DMEM (Sigma-Aldrich, St. Louis, Mo.) plus 10% FBS. The mixture was centrifuged at 250g, and the cells were resuspended in DMEM plus 10% FBS and a 1% ampicillin/streptomycin mixture (Sigma-Aldrich, St. Louis, Mo.) and then were plated in a 75-cm^2 flask (BD Biosciences, Bedford, Mass.) at a concentration of 10–15 $\times$ 10^3 cells/cm^2. Cells were cultured for 24 hours at 37° in an atmosphere of 5% CO_2 in air. Then the dishes were washed with PBS to remove nonadherent cells and cell fragments. The cells were maintained in culture in the same medium and under the same conditions until they reached approximately 80% confluence, with replacement of the culture medium every 3 to 4 days. Cells were then passaged with 0.05% trypsin-EDTA (Gibco, Invitrogen Corporation, Carlsbad, Calif.) at a dilution of 1:3. We used these cells (between passages 1 and 3) as ADSCs for transplantation, and characterization of ADSCs was performed using cells at passages 1 to 3. Furthermore, doubling time was determined by manually counting the number of cells.

In Vitro Differentiation of ADSCs

Adipogenic Differentiation

Adipogenic differentiation was performed by the method previously described by Lee et al[4] with slight modifications. To induce adipogenic differentiation, ADSCs were seeded at a density of 10^4 cells/cm^2 in 8-chamber slides (Nunc, Inc., Naperville, Ill.) and cultured in α-MEM (Nacalai Tesque, Kyoto, Japan) + 10% FBS until reaching 100% confluence; thereafter, the cells were further cultured for 21 days in the presence of 1 μM dexamethasone (Sigma-Aldrich, St. Louis, Mo.), 5 μg/mL recombinant human insulin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 4.5 g/L glucose (Wako Pure Chemical Industries, Ltd., Osaka, Japan), as reagents for adipogenic differentiation. The cells cultured without these reagents (α-MEM + 10% FBS alone) served as a negative control. Adipogenic differentiation was confirmed by the formation of neutral lipid vacuoles stained with Oil Red O (Wako Pure Chemical Industries, Ltd., Osaka, Japan). For the Oil Red O stain, cells were fixed with 10% formalin, washed, and stained with a working solution of 0.18% Oil Red O for 5 minutes. The nuclei were counterstained with Mayer's hematoxylin solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Osteogenic Differentiation

To promote osteogenic differentiation, ADSCs were seeded at a density of 1 $\times$ 10^4 cells/cm^2 in 8-chamber slides and cultured in DMEM + 10% FBS until they reached 70% to 80% confluence. Osteogenic differentiation of ADSCs was induced by culturing them for 4 weeks with the osteogenic induction medium, which consisted of 0.1 μM dexamethasone (Sigma-Aldrich, St. Louis, Mo.), 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, Mo.), and 0.2 mM ascorbate (Sigma-Aldrich, St. Louis, Mo.).[7] The cells cultured without these reagents (DMEM + 10% FBS alone) served as a negative control. Osteogenic differentiation was confirmed by the increased expression of alkaline phosphatase (ALP) by histochemical staining (TRACP & ALP double-stain kit; TAKARA BIO, Otsu, Shiga, Japan) and also by von Kossa

staining determining the deposition of the hydroxyapatite matrix.

Neural Differentiation

Neural differentiation of ADSCs was carried out according to the method described elsewhere (Woodbury et al, 2000).[8] Briefly, subconfluent ADSCs (70%–80% confluence) were cultured for 24 hours in preinduction medium [DMEM, 20% FBS, 1 mM β-mercaptoethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan)], and the cells were further incubated in the neurogenic medium (NM), which consisted of DMEM and 10 mM β-mercaptoethanol.[9] Neural differentiation was immunohistochemically determined by the expression of neural-specific enolase (NSE; BIOMOL International, Butler Pike Plymouth Meeting, Penn.).

Epithelial Differentiation

For epithelial differentiation, ADSCs were incubated with ATRA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at a final concentration of 5 μM as previously described by Brzoska et al.[10] The cells incubated without ATRA [the equivalent volume of DMSO (Nacalai Tesque, Kyoto, Japan), solvent for ATRA, was added to the culture] served as a negative control. The medium was replaced every 2 days during a total incubation period of 10 days. Differentiation was immunohistochemically and flow-cytometrically determined by the expression of cytokeratin-18 because of the preferential expression of the "primary" keratins on the intestinal epithelia.

Flowcytometry

Freshly isolated undifferentiated ADSCs (passages 0–3) were stained with unconjugated FITC- or PE-labeled monoclonal antibody (mAb) against CD11b, CD45, or CD90 (CALTAG Laboratories, Invitrogen Corporation, Carlsbad, Calif.), CD31 (Becto-Dickinson, Franklin Lakes, NJ), CD34 (Santa Cruz Biotechnology, Inc. Santa Cruz, Calif.). FITC-labeled goat antimouse IgG (BD Biosciences, San Jose, Calif.) was used as secondary antibody when necessary. In the case of staining with anti-cytokeratin-18 mAb, cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences, San Jose, Calif.). The cells thus treated were intracytoplasmically stained with FITC-anti-cytokeratin-18 Ab (PROGEN Biotechnik, Heidelberg, Germany). The stained cells were analyzed by a FACScan (BD Bioscience, San Jose, Calif.).

Measurement of HGF, VEGF, TGF-β, and Adiponectin

ADSCs were cultured in standard 12-well plates until 80% confluence and then culture supernatant was collected. The concentration of HGF (Institute of Immunology Co., Ltd., Tokyo, Japan), VEGF (R&D Systems, Minneapolis, MN), TGF-β (BioSource International, Inc., Camarillo, Calif.), and adiponectin (AdipoGen, Inc., Seoul, Korea) was measured by ELISA according to the manufacturers' instructions.

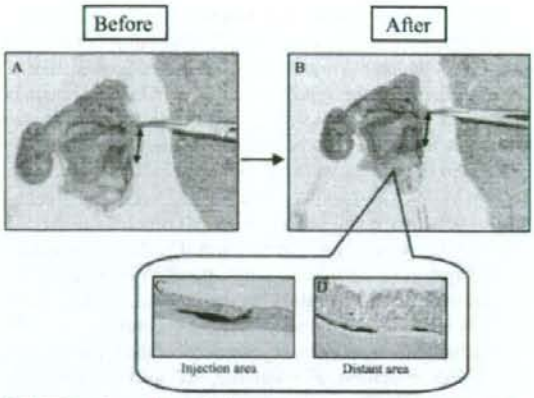

FIGURE 1. Procedure of submucosal injection. A: Under diethyl ether anesthesia, the intestine was exposed by a midline incision of the abdomen (blue arrow indicates the ulcer area injured by the TNBS injection) B: ADSCs or PBS (negative control) were injected from the serosa side into the submucosal layer. C: Using India ink, we confirmed that ADSCs were actually injected into the submucosal layer by this method. D: India ink was found in the submucosal layer of the area distant from the injection point.

In Vivo Examination

Induction of Colitis by TNBS

Colitis was induced by TNBS using the method described previously.[11] Briefly, rats were anesthetized after a 24-hour fast. Then an infant tube (indwelling feeding tube for infants, 4Fr, diameter = 1.35 mm; Atom Medical Co., Tokyo, Japan) was inserted into the anus, and the tip was advanced to 6 cm proximal in the colon. TNBS (Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in 50% ethanol was instilled into the colon through the cannula (30 mg of TNBS in a volume of 0.5 mL). After the instillation, the rats were held upside down by their tails for 60 seconds and then returned to their cages. We prepared more than 40 rats, and colitis was observed in all the rats injected with TNBS. Similarly, rats instilled with PBS served as controls (n = 10). All rats were sacrificed 10 days after administration of TNBS.

Submucosal Injection of ADSCs

ADSCs (passages 0–3), harvested from the culture by trypsin/EDTA solution, were suspended at 10^7 cells in 0.5 mL of PBS containing 2% FBS. Under diethyl ether anesthesia, the intestine was exposed by a midline incision of the abdomen, and ADSCs (10^7 cells) were injected from the serosa into the submucosa of the colon 2 days after the TNBS injection (n = 10; Fig. 1). Rats injected with 0.5 mL of PBS containing

2% FBS served as controls (n = 10).

Furthermore, using India ink, we confirmed that ADSCs were actually injected into the submucosal layer by this method, and India ink was found in the submucosal layer of 9 of the 10 rats injected with India ink (90%). It is noted that no complications were observed after the injection of 0.5 mL of ADSCs or PBS into the submucosa.

Assessment of Inflammation in TNBS-Induced Colitis

To examine the severity of colitis, the body weight of the treated rats was measured every other day, and clinical findings such as area of ulcer (measured using NIH image software on the pictures of colon), length (colocecal junction to anal verge), and weight of the colon 10 days after the TNBS injection were also assessed.

Histological Examination

The tissue specimens were fixed in buffered formalin and embedded in paraffin, and tissue sections were stained by H&E. Colonic inflammation was assessed using modification of the histopathologic grading system of Macpherson and Pfeiffer[12,13]: grade 0 = normal findings; grade 1 = mild mucosal and/or submucosal inflammatory infiltrate (admixture of neutrophils) and edema, punctate mucosal erosions often associated with capillary proliferation, muscularis mucosae intact; grade 2 = grade 1 changes involving 50% of the specimen; grade 3 = prominent inflammatory infiltrate and edema (neutrophils usually predominating), frequently with deeper areas of ulceration extending through the muscularis mucosae into the submucosa, rare inflammatory cells invading the muscularis propria but without muscle necrosis; grade 4 = grade 3 changes involving 50% of the specimen; grade 5 = extensive ulceration with coagulative necrosis bordered inferiorly by numerous neutrophils and lesser numbers of mononuclear cells, necrosis extends deeply into the muscularis propria; grade 6 = grade 5 changes involving 50% of the specimen. All scoring was performed by the same individual under blind conditions.

Measurement of Myeloperoxidase Activity

Tissue myeloperoxidase (MPO) activity was determined by a standard enzymatic procedure as previously described by Krawisz et al[14] with slight modifications. Total protein concentrations of the tissue supernatant and whole-cell lysate were measured using a BCA Protein Assay Kit (PIERCE Co., Rockford, Ill.) for calibration, and myeloperoxidase activity in the tissue homogenate was determined using a Myeloperoxidase Assay Kit (CytoStore Inc., Calgary, Alberta, Canada) according to the manufacturer's instructions.

Measurement of Cytokines in Colonic Tissue

The colonic tissue was homogenized in cold PBS using a Polytron-type homogenizer. Tissue homogenate was then centrifuged at 20,000g for 20 minutes at 4°C to obtain the supernatant. Total protein concentrations of the tissue supernatant and whole-cell lysate were measured using a BCA Protein Assay Kit (PIERCE Co., Rockford, Ill.) for calibration, and protein concentrations of IL-1β (BioSource International, Inc., Camarillo, Calif.), GRO/CINC-1 (functionally equivalent to IL-8; Panafarm Laboratory, Kumamoto, Japan), TNF-α (BioSource International, Inc., Camarillo, Calif.), and IFN-γ (BioSource International, Inc., Camarillo, Calif.) in the tissue homogenate were determined using ELISA kits according to the manufacturers' instructions.

In Situ Cell Proliferation

To evaluate proliferation of the colonic epithelium, 50 mg/kg of 5-bromo-2'-deoxyuridine (BrdU; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was injected intraperitoneally 1 hour before sacrifice, and cells synthesizing DNA were immunohistochemically identified using a BrdU In-Situ Detection Kit (BD Biosciences, San Jose, Calif.) according to the manufacturer's instructions. We then counted the BrdU-positive cells in 10 crypts of the mucosa-bordering ulcer.

Y-Chromosome Fluorescence in Situ Hybridization (Y-FISH)

For assessment of distribution of ADSCs injected into the submucosa, in vivo experiments were performed in sex-mismatched conditions. TNBS-induced colitis models were prepared in female rats, and ADSCs obtained from male rats were injected into the submucosa of the colon in TNBS-induced colitis models. To detect the Y chromosome of ADSCs, we used a STAR-FISH Rat 12/Y Paint (Y FITC; 12 biotin) probe purchased from Cambio (Dry Drayton, Cambridge, UK), and detection protocols were conducted with a Histology FISH Accessory Kit (Dako Cytomation, Dako, Denmark).

Immunofluorescent Staining

Following Y-FISH, colonic tissues in paraffin-embedded sections (3 μm) were stained with mouse mAb against pan-cytokeratin (CHEMICON, now part of Millipore Corporation, Billerica, Mass.), vimentin (Santa Cruz Biotechnology, Inc. Santa Cruz, Calif.), S-100 (COSMO Bio Co., Ltd., Tokyo, Japan) or SMA (Lab Vision Corporation, Fremont, Calif.) after pretreatment procedure (epitope retrieval). They were then stained with PE-labeled goat antimouse IgG antibody (BD Biosciences, San Jose, Calif.). Cellular nuclei were counterstained with DAPI. Images were captured by a LSM510-META and exported as TIFF files and further processed in Adobe PhotoShop.

Statistical Analysis and Ethical Considerations

Results are expressed as mean ± SDs. Differences between groups were examined for statistical significance using the