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Toshiyuki Yamane, Takahiro Kunisada, Hirotake Tsukamoto, Hidetoshi Yamazaki, Hitoshi Niwa, Shinji Takada, and Shin-Ichi Hayashi

Hemopoietic cells develop in a complex milieu that is made up of diverse components, including stromal cells. Wnt genes, which are known to regulate the fate of the cells in a variety of tissues, are expressed in hemopoietic organs. However, their roles in hemopoiesis are not well characterized. In this study, we examined the roles of Wnt proteins in hemopoiesis using conditioned medium containing Wnt-3a. This conditioned medium dramatically reduced the production of B lineage cells and myeloid lineage cells, except for macrophages in the long-term bone marrow cultures grown on stromal cells, although the sensitivity to the conditioned medium differed, depending on the hemopoietic lineage. In contrast, the same conditioned medium did not affect the generation of B lineage or myeloid lineage cells in stromal cell-free conditions. These results suggested that Wnt proteins exert their effects through stromal cells. Indeed, these effects were mimicked by the expression of a stabilized form of β-catenin in stromal cells. In this study, we demonstrated that Wnt signaling regulates hemopoiesis through stromal cells with selectivity and different degrees of the effect, depending on the hemopoietic lineage in the hemopoietic microenvironment. The *Journal of Immunology*, 2001, 167: 765–772.

B one marrow (BM) is the major site of hemopoiesis in the adult, and supports both myeloid and lymphoid lineage development (1, 2). The spleen also retains the capacity to expand hemopoietic cells, particularly in the mouse, although this organ does not support the development of lymphoid lineages (3, 4). Hemopoietic microenvironments in these organs permit hemopoietic populations to self-renew and generate maturing cells. Stromal cells are major components of such microenvironments (5, 6). The term “stromal cells” is used to designate nonhemopoietic tissue cells of such organs, and does not imply a single cell type. Rather, the term designates heterogeneous populations, including reticular cells, fibroblasts, adipocytes, and endothelial cells (6). Stromal cells support hemopoiesis by providing cytokines, chemokines, adhesion molecules, and extracellular matrix molecules. Many laboratories have established stromal cell clones that support hemopoiesis (5). Such clones appear to be fibroblast, preadipocyte, or endothelial cell lines. In vitro culture systems using these clones have contributed to understanding blood cell production by revealing important cell-cell or cell-matrix interactions, and essential soluble mediators.

Recent reports suggested that Wnt proteins might participate in the regulation of blood cell production (7–9). Wnt genes encode a large family of secreted glycoproteins that are involved in a wide spectrum of cell activities in development (10). Wnt proteins are thought to be associated with the cell surface or extracellular matrix of the secreting and adjacent cells (11). Wnt proteins transduce signals through their receptors, Frizzled and Disheveled, leading to the inactivation of GSK3β, which normally keeps the level of β-catenin low in the absence of signals through interacting with the product of the adenomatous polyposis coli gene and Axin. In response to the Wnt signal, β-catenin accumulates, interacts with the lymphoid enhancer factor-1/T cell factor-1 family of transcription factors, and activates the target genes (10). In the mouse, 18 different Wnt genes have been reported. At least three Wnt genes are expressed in hemopoietic tissues in the mouse. In fetal hemopoietic sites, the Wnt-5a and Wnt-10b genes are expressed (7). In the BM, the expression of the Wnt-5a gene in addition to the Wnt-5a and Wnt-10b genes has been reported (9). These Wnt genes are also known to be involved in several aspects of embryogenesis (12–16). Functions of Wnt proteins in hemopoiesis have been examined using culture supernatants, retrovirus-mediated gene transfer, or coculture with Wnt gene-transfected cells, because the purification of biologically active Wnt proteins has not yet been successful. In the mouse, the potential of Wnt-1, -5a, and -10b to expand fetal liver hemopoietic progenitors, and of Wnt-3a to augment the proliferation of pro-B cells, has been reported (7, 9).

These experiments have demonstrated that Wnt proteins stimulate the survival and proliferation by acting directly on hemopoietic progenitors. However, the effects on hemopoietic microenvironments must be further investigated to fully understand the role of molecules regulating hemopoiesis. Indeed, stromal cells express Frizzled receptors and respond to Wnt proteins, as we show in this work. In this study, we examined the effects of Wnt signaling on hemopoiesis, including the effects on stromal cells, using the conditioned medium (CM) from L cells secreting Wnt-3a (17). In addition, an inducible expression system of a stable form of β-catenin in stromal cells was established to mimic the effect of the

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1 Abbreviations used in this paper: BM, bone marrow; CFC, colony-forming cell; CM, conditioned medium; DEX, dexamethasone; HA, hemagglutinin; 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; OPG, osteoprotegerin; RANKL, receptor activator of NF-κB ligand; Tc, tetracycline; TRAP, tartrate-resistant acid phosphatase; CFU-G, CFU-granulocyte.
REGULATION OF HEMOPOIESIS BY Wnt SIGNALING

Wnt signaling. By using these tools, we demonstrate that Wnt signaling negatively regulates hematopoiesis, but with selectivity and different sensitivities of different hematopoietic lineages in the presence of stromal cells.

Materials and Methods

Mice

Female 6- to 10-wk-old (C57BL/6 × DBA/2J, mice were purchased from Japan SLC (Hamamatsu, Japan). BM cells were prepared from the femora and tibiae of these mice.

Cell lines

ST2 (18), a BM-derived stromal cell line, was maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 5% FCS (Bio-Whittaker, Walkersville, MD) and 50 μM 2-ME. ST2b cells were maintained in the same medium, except that it was supplemented with 1 μg/ml tetracycline (Tc).

Conditioned medium

Wnt-3a-producing L cells and control L cells (17) were grown to confluency in DMEM/F-12 medium (Life Technologies) supplemented with 10% FCS. Then the medium was replaced with α-MEM supplemented with 10% FCS, and the culture supernatants were collected after 24 h of culturing. These conditioned media were designated W3a-L-CM and L-CM, respectively. To collect the culture supernatants of L cells stimulated with Wnt-3a, confluent control L cells were cultured with 25% W3a-L-CM for 48 h, and washed three times with PBS, and the medium was changed to α-MEM supplemented with 10% FCS. The culture supernatants harvested 24 h later were designated as L824-L-CM. These conditioned media were added to cultures at the proportion of 25% in all experiments reported in this work (17).

Tc-regulated system

ST2 cells were first transfected with a construct that expressed the Tc-controlled transactivator-internal ribosomal entry site-hph gene under the control of the CAG promoter (Refs. 19 and 20; H. Niwa, unpublished observations), and cultured in the presence of 250 μg/ml hygromycin B (Sigma, St. Louis, MO). Resistant cells were further cotransfected with a construct that expressed a stabilized form of mouse β-catenin (21), which was tagged with the hemagglutinin (HA) epitope at the carboxy-terminal (a gift from A. Nagafuchi, Kumamoto University, Kumamoto, Japan) under the control of the human CMV-IE promoter (Tc-responsive promoter (19) and a PGK-neo+ construct, and selected in the presence of 200 μg/ml G418 (Life Technologies), 25 μg/ml hygromycin B, and 1 μg/ml Tc (Sigma). Transfections were performed with GenePORTER (Gene Therapy System, San Diego, CA). The stable transfectants, designated as ST2b, were maintained in the presence of 1 μg/ml Tc. Experiments were performed in the range of 0–100 ng/ml Tc because 1 μg/ml Tc slightly suppressed osteoclast formation on parental ST2 cells, and 100 ng/ml Tc was sufficient to block the expression of transfected β-catenin.

Long-term BM cultures

For the long-term BM culture of myeloid lineage cells (Dexter-type culture) (22), 106 BM cells were seeded onto ST2 or ST2b cells in 60-mm dishes (Corning Glass, Corning, NY) gelatin-coated dishes, or PRIMA-MEM (Amersham Pharmacia Biotech). In some experiments, the membranes were repropulated with mouse anti-α-actin Ab (Sigma) or rabbit anti-HA-tag Ab (Medical & Biological Laboratories, Nagoya, Japan), then incubated with HRP-labeled secondary Abs, and detected using the same system.

Immunofluorescent staining

ST2 cells were fixed with 4% paraformaldehyde, 0.18% Triton X in PBS for 10 min at room temperature, blocked with 1% BSA in PBS, incubated with mouse anti-β-catenin Ab (Transduction Laboratories, Lexington, KY). Immunoreactive bands were visualized using the ECL detection system (Amersham Pharmacia Biotech). In some experiments, the membranes were repropped with mouse anti-α-actin Ab (Sigma) or rabbit anti-HA-tag Ab (Medical & Biological Laboratories, Nagoya, Japan), then incubated with HRP-labeled secondary Abs, and detected using the same system.

PCR analysis

Total RNA was purified using ISOGEN (Nippon Gene, Toyama, Japan). First-strand cDNA synthesis was performed using ReverseTracer DNA (Toyobo, Osaka, Japan) primed with random hexamer in a 20-μl reaction mixture containing 1 μg total RNA. One microliter of the first-strand cDNA mixture was subjected to PCR with Taq polymerase (Toyobo) in a 25-μl volume. The PCR conditions were as follows: 94°C (5 min), 55°C (2 min), 72°C (3 min) for the primary cycle; 94°C (1 min), 55°C (1 min), 72°C (1.5 min) for the following 30 cycles. The extension time in the last cycle was 4.5 min. The following primers were used: Fzdz1 (GenBank accession number AF015462), 5'-GTA CTGAGCGCCGGATGTGTCTCT-3' / 5'-GATCATGGAAGCTGTTAGAATCT-3'; Fzdz3 (U33205), 5'-TTTAAAGAAATCAAGAGAAGACTG-3' / 5'-TACTGAACCTCAGTCTGCTGGT-3'; Fzdz4 (U33317), 5'-GAACTGACTCGTGTAGTCTG-3' / 5'-GTACGCTGCTGTCACG-3'; Fzdz5 (AP02523), 5'-GCAGAGGACCGAAGACAGTCCAC-3' / 5'-ACCGTGAC AAGACCGAGGCA-3'; Fzdz6 (U33349), 5'-TGTCGCACTGATCCAGCTGATC-3' / 5'-GCTGATGGAAGCAGGAGAGATGTTG-3'; Fzdz7 (U33320), 5'-AGTTGGCTACGTGGCGCGTGCTCTGT-3' / 5'-TGATGAGCCCGACGTAG.
GGTCATCA-3'; Fzd8 (U43321), 5'-CGATTACGGCTCTCATGCTCAAG 3'/5'-AAGATACAGAGCTGGCCGTGCCAGA-3'. Identities of the amplified fragments and the reported sequences were confirmed by digestion with restriction enzymes.

Results

Wnt signaling components in stromal cells

We found that the CM from L cells secreting Wnt-3a (W3a-L-CM), but not the control CM (L-CM), causes a morphological change of the ST2 stromal cell line: the ST2 cells overgrow and form highly condensed sheets of cells (Fig. 1A). The growth rate of ST2 cells seems not to be changed, but the cells grow even after they have reached confluency and the cell number increases to ~2.5-fold that of control cultures (data not shown). These changes are similar to those observed in Wnt gene-transfected mammary epithelial cells (29). We examined the components of Wnt signaling in stromal cells. Expression of murine frizzled homologues (Fzd1, 3, 4, 5, 6, 7, 8), the receptors for Wnt proteins, was examined by RT-PCR analysis. The expression of all of these Fzd genes, except for Fzd3, was observed in ST2 cells (Fig. 1B). When ST2 cells or primary BM stromal cells were stimulated by W3a-L-CM, the concentration of cytosolic β-catenin dramatically increased (Fig. 1C). Translocation of β-catenin to the nucleus was also observed (Fig. 1D). These results suggest that Wnt signaling components are functional in stromal cells.

Effects of W3a-L-CM on hemopoiesis

We examined the effects of W3a-L-CM on in vitro cultures of hemopoietic lineages cultured on stromal cells. We prepared the Dexter-type cultures for the myeloid lineage cells on ST2 in the presence of L-CM or W3a-L-CM. ST2 cells were treated with mitomycin C to avoid indirect effects of their growth. Under both conditions, the majority of the cells that adhered to ST2 were macrophages (Fig. 2, C and D). Perhaps this was due to the high dose of M-CSF in the CM of L cells (30). About half the number of cells were harvested from the culture containing W3a-L-CM compared with the culture with L-CM. We analyzed these cells by flow cytometry. The number of macrophages, which were identified by their sensitivity to antagonistic Ab to the receptor for M-CSF (AFS98), was not changed much by the addition of W3a-L-CM, but the AFS98-resistant Mac-1/Gr-1 granulocyte population was clearly reduced by adding W3a-L-CM (Fig. 3). We next prepared Dexter-type cultures on mitomycin C-treated ST2 in the presence of AFS98 to avoid the predominance of macrophages in the culture. In the cultures supplemented with L-CM, hemopoietic colonies firmly attached to ST2 were observed (Fig. 2A). In contrast,
there were only small colonies loosely attached to ST2 in the cultures containing W3a-L-CM (Fig. 2B). Hemopoietic colonies were never observed beneath the stroma under these conditions. Only about one-twentieth of the number of cells was recovered from the culture with W3a-L-CM compared with the control cultures (Fig. 4A). The numbers of mature neutrophils and mast cells in the W3a-L-CM-containing cultures were reduced to ∼5% and 10% of those in the cultures with L-CM, respectively, as assessed by May-Grünwald-Giemsa staining (Fig. 4A). The numbers of CFCs in these cultures, examined in semisolid methylcellulose-containing media, were also diminished to ∼2.5% of those in the control cultures (Fig. 4A). Because L cells themselves can respond to W3a-L-CM (17), the possibility remained that Wnt-3a produced by Wnt-3a-transfected L cells acts directly on these L cells and changes the contents of the culture supernatant compared with that of the parental L cells. Such a difference of contents might indirectly affect the results observed in the culture to which W3a-L-CM was added. To exclude such a possibility, we collected the CM of L cells that had been stimulated with W3a-L-CM for 48 h and cultured for an additional 24 h after washing. In these CM, L cells, high levels of cytosolic β-catenin last for at least 12 h after washing (data not shown). This CM, designated as L W3a-CM, did not cause effects distinct from those of L-CM in Dexter-type cultures (Fig. 4A), suggesting that the dramatic changes observed in these cultures were caused by Wnt proteins.

Next, the effects of W3a-L-CM on Whitlock-Witte-type cultures for B lymphopoiesis were examined on mitomycin C-treated ST2. Although B lymphopoiesis proceeded normally in the cultures supplemented with L-CM, no colonies of the B lineage were observed when W3a-L-CM was added (Fig. 2, E and F). The number of cells recovered in W3a-L-CM-supplemented cultures was only about one-fifth of that recovered from the control (Table I). Furthermore, when these cells were analyzed by flow cytometry, B220⁺ cells in W3a-L-CM-supplemented cultures constituted only 2.3% of the population, and the total number of B220⁺ cells per well was reduced to 0.6% of that in the cultures with L-CM (Table I). About a 2-fold increase of the number of Mac-1⁺ macrophages was observed in W3a-L-CM-supplemented cultures (Table I). These effects of W3a-L-CM were not found with L W3a-CM (Table I).

The effect of W3a-L-CM on osteoclastogenesis of BM cells was also examined on mitomycin C-treated ST2. In these cultures, two essential cytokines for osteoclastogenesis, M-CSF and RANKL (also known as osteoprotegerin ligand, osteoclast differentiation factor or TNF-related activation-induced cytokine), are provided by ST2 cells (31–33). M-CSF is constitutively produced by ST2, and RANKL is expressed by ST2 that have been treated with 1.25(OH)₂D₃ and DEX (32). When W3a-L-CM, but not L W3a-CM, was added, the number of TRAP-positive osteoclasts generated was reduced (Fig. 4B, left). The degree of reduction of osteoclasts fluctuated highly from experiment to experiment, resulting in the generation of from 0.8% to 42% of the number in the cultures treated with L-CM.

To discriminate whether these inhibitory effects of W3a-L-CM on hemopoiesis are direct effects on hemopoietic cells or indirect effects exerted through stromal cells, we performed stromal cell-free culture for osteoclasts and the colony formation assay in methylcellulose for other lineages using BM cells. Sephadex G-10 column-purified BM hemopoietic cells were induced to differentiate into osteoclasts with M-CSF and soluble RANKL under stromal cell-free conditions in the presence of W3a-L-CM or L-CM. Under such conditions, no inhibitory effect of W3a-L-CM was observed.

### Table 1. Effects of Wnt signaling on B lymphopoiesis

<table>
<thead>
<tr>
<th>Strona</th>
<th>Conditions</th>
<th>No. of Cells Recovered (×10⁵)</th>
<th>B220⁺ %</th>
<th>B220⁺ %</th>
<th>Mac-1⁺ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST2</td>
<td>L-CM</td>
<td>22.0 ± 2.0</td>
<td>67.9</td>
<td>10.9</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>W3a-L-CM</td>
<td>4.7 ± 0.6</td>
<td>2.1</td>
<td>0.2</td>
<td>93.9</td>
</tr>
<tr>
<td></td>
<td>L W3a-CM</td>
<td>18.7 ± 1.5</td>
<td>70.3</td>
<td>6.4</td>
<td>7.9</td>
</tr>
<tr>
<td>ST2b</td>
<td>Te⁺</td>
<td>24.9 ± 3.0</td>
<td>87.8 ± 1.9</td>
<td>10.7 ± 1.7</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Te⁻</td>
<td>32.1 ± 2.8</td>
<td>90.0 ± 1.7</td>
<td>8.8 ± 1.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Triplicate cultures grown on mitomycin C-treated ST2 were pooled and analyzed. Triplicate cultures on ST2b were analyzed independently by flow cytometry.
(Fig. 4B, right), suggesting that W3a-L-CM controls osteoclastogenesis through stromal cells.

To clarify the basis of these results, we conducted Northern hybridization analysis for M-CSF, RANKL, and osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor, a decoy receptor for RANKL that is also known to be produced by stromal cells (34, 35), to examine whether the reduction of osteoclasts is merely attributable to the expression levels of these molecules. Analyses using an image analyzer suggested that W3a-L-CM changes the expression levels of RANKL and OPG in ST2 cells in the presence of 1,25(OH)2D3, and DEX to about one-half and 1.6-fold the levels in control L-CM, respectively. However, these changes of the expression levels of these factors could not fully explain the reduction of osteoclasts, because the addition of an excess amount of RANKL to the culture only partially rescued the osteoclastogenesis (data not shown).

We also examined the direct effects of W3a-L-CM on hemopoietic cells by adding it to colony formation assays. As shown in Fig. 4C, no inhibitory effect of W3a-L-CM on the colony-forming activity (CFU-granulocyte (CFU-G), CFU-macrophage, CFU-granulocyte-macrophage, burst-forming unit-erythroid, and CFU-IL-7) was observed. The size of colonies was also similar in W3a-L-CM- and L-CM-supplemented cultures. Also, the colonies derived from CFU-granulocyte-macrophage and CFU-G contained normal mature neutrophils (data not shown). These results suggest that W3a-L-CM inhibits hemopoiesis through the stromal cells.

Establishment of a Tc-regulatory system to express β-catenin in ST2

The experiments described above strongly suggested that Wnt proteins might control hemopoiesis through stromal cells. However, the following possibilities remained: 1) Wnt-3a might signal hemopoietic cells directly, and generate phenotypes only when stromal cell-derived factors are present. 2) Wnt-3a and other factors in the CM of L cells might act synergistically on stromal cells. To overcome these problems and avoid the difficulty of purifying Wnt proteins with biological activity, we established ST2 cells that constitutively express a Tc-controlled transactivator (19) and express a stabilized form of β-catenin (21) under the control of the human CMV*1 promoter, a Tc-responsive promoter (19). In these cells (named ST2b), the expression of the stabilized form of β-catenin is induced in the absence of Tc, mimicking the state of ST2 cells stimulated with Wnt proteins (21). As described in Fig. 5A, the expression of the introduced β-catenin, which is tagged with the HA epitope and detected as a band shifted relative to endogenous β-catenin, was induced by the decrease of Tc in a dose-dependent manner from 5 to 0 ng/ml. When the expression of the stabilized β-catenin was induced by the removal of Tc, overgrowth of ST2b was observed, along with an increase of the β-catenin level in these cells (Fig. 5, B and C). However, such changes were observed 1 wk after the cells reached confluency in medium containing 10% FCS, but not in 5% FCS, although the effects of W3a-L-CM are detected immediately after the cells reach confluence, and these effects are detected even in 5% FCS. This might be explained by the fact that cytosolic β-catenin levels are significantly higher when cells are stimulated with W3a-L-CM (compare Figs. 1C and 5A). In any case, up-regulation of the cytosolic β-catenin level in stromal cells causes a phenotype similar to the state induced by W3a-L-CM.

Regulation of hemopoiesis-supporting ability of stromal cells by β-catenin

We next investigated whether the expression of stabilized β-catenin changes the ability of stromal cells to support the development of hemopoietic lineages. We conducted Dexter-type culture on ST2b cells for the myeloid lineage cells. In the absence of Tc, no change of the number of macrophages was detected (data not shown). However, the numbers of neutrophils and mast cells were reduced to ~7–16% and 29–38% of those in Tc-containing cultures, respectively (Fig. 6A). Hemopoietic colonies on ST2b cells seemed to be reduced in size when β-catenin was induced (Fig. 6B). In contrast to this appearance, these cultures contained equivalent numbers of cells that formed colonies in methylcellulose, as shown in Fig. 6A, suggesting that changes of the adhesive properties cause such progenitors to become detached from stromal cells. Because ST2b cells overgrew in Dexter’s conditions upon the removal of Tc, we examined whether these phenotypes were also reproducible even when ST2b cells were mitotically inactivated with mitomycin C. The reduction of neutrophils and mast cells was observed even in these conditions, suggesting that the changes of the hemopoiesis-supporting ability of stromal cells were not secondary effects of their overgrowth (Fig. 6E, data not shown). The modest reduction compared with that in untreated cultures might be explained by the lower level of induction of β-catenin in mitomycin C-treated cultures. The generation of osteoclasts was also affected by the induction of stabilized β-catenin. The number of TRAP+ multinucleated osteoclasts produced was reduced to 49–66% of that in the culture containing Tc (Fig. 6D).
In contrast, no significant changes of B lymphopoiesis were observed when the expression of the stabilized β-catenin was induced in ST2b in Whitlock-Witte-type cultures in the presence of IL-7. These B lineage cells also differentiated normally to the μ-chain + stages whether the stabilized β-catenin was induced or not (Fig. 6C and Table I).

Discussion
In this study, we have demonstrated that Wnt protein acts on stromal cells and controls their functions. Stromal cells express frizzled genes, and cytosolic β-catenin is stabilized and translocated to the nucleus upon the stimulation with Wnt3a-L-CM. W3a-L-CM promoted the growth of ST2 cells after they reached confluence. ST2b cells also overgrew 1 wk after the induction of a stabilized form of β-catenin by removal of Tc. Thus, we showed that stromal cells can respond to Wnt signaling and are candidate targets of Wnt proteins in hemopoietic microenvironments.

Wnt proteins are also known to act directly on hemopoietic cells (7–9). We observed the stabilization of β-catenin in Sephadex G-10 column-purified BM hemopoietic cells and several hemopoietic cell lines stimulated with W3a-L-CM (data not shown). However, the following results support the view that Wnt proteins regulate hemopoiesis more markedly through stromal cells. First, W3a-L-CM suppressed the long-term BM cultures (Dexter’s type-culture of the myeloid lineages and Whitlock-Witte-type culture of the B lineage cells) on ST2. In contrast, W3a-L-CM did not suppress the colony formation in semisolid media. Second, W3a-L-CM inhibited osteoclastogenesis in the presence of stromal cells, but not in absence of stromal cells. And finally, induced expression of a stabilized form of β-catenin in stromal cells, although not completely, mimicked the effects of W3a-L-CM.

Different degrees of reduction of various hemopoietic lineages were caused by the addition of W3a-L-CM in the presence of ST2 cells. The production of B lineage cells was most severely affected: W3a-L-CM reduced the number of B220 + cells by >99%. The numbers of CFCs, neutrophils, and mast cells were reduced to ~2.5%, 5%, and 10% of those in L-CM-treated cultures, respectively. The number of osteoclast lineage cells was reduced to 0.8–40% of the number in the control cultures. In contrast, the generation of macrophage lineage cells was not affected much. These results suggested that the responsiveness and sensitivities differ among the various hemopoietic lineages.

Upon inducing the expression of a stabilized form of β-catenin by removal of Tc from the cultures, the generation of neutrophils, mast cells, and osteoclasts was diminished to 12%, 34%, and 58% (on average) of the levels generated in Tc-containing cultures, respectively. The generation of macrophages was not affected by the expression of this stabilized form of β-catenin. These patterns of selectivity and sensitivity were similar to those obtained with W3a-L-CM. However, B lymphopoiesis and the generation of CFCs were not affected, contrary to the effects of W3a-L-CM addition.

This difference might result from differences of the β-catenin levels, because W3a-L-CM induced higher levels of β-catenin compared with the Tc-regulated system. This notion might be supported by the fact that the reduction of neutrophils, mast cells, and osteoclasts was more severe when W3a-L-CM was added, and that W3a-L-CM induced changes of cell growth more rapidly than the Tc-regulated system. The following possibilities also remain: 1) W3a-L-CM might control B lymphopoiesis and generation of CFCs synergistically with other factors produced by L cells; 2) some effects of W3a-L-CM might be due to factors other than Wnt-3a that are induced and secreted into the culture medium of Wnt-3a-transfected L cells upon the stimulation with Wnt-3a produced by themselves; 3) Wnt signaling pathways not mediated through the stabilization of β-catenin are responsible (10); or 4) W3a-L-CM might act directly on the hemopoietic cells. The second possibility might be ruled out by our experiments using L.Wnt 3a-CM. The interpretation of our results according to the last possibility generates the view contrary to others because it has been reported that Wnt proteins promote, rather than suppress, the proliferation of multipotent and committed hemopoietic progenitors (7, 8), and that the same W3a-L-CM we used promotes the proliferation of fetal liver pro-B cells (9). However, we could not exclude this possibility. As the reported experiments were performed in suspension cultures, the effect of W3a-L-CM on molecules involved in hemopoietic cell-stromal cell interactions must also be discussed. For B lymphopoiesis, we have to take it into consideration that we used adult BM cells, while Reya et al. (9) used fetal liver cells. We must...
also pay attention to the fact that CFU-IL-7 and hemopoietic progenitors that give rise to B lineage cells in Whittlock-Witte culture are in different stage of development (25). Although we demonstrated that the colony-forming activity of CFU-IL-7 in methylcellulose was unaffected by the addition of W3a-L-CM, the possibility remains that W3a-L-CM directly suppressed B progenitors in earlier stage. We have to conduct more detailed analyses for the last possibility.

In contrast to our results, it was reported that the CM of 293 cells containing Wnt-5a proteins expands the myeloid and lymphoid CFCs even in cocultures with stromal cells (7). This might be due to differences of the properties of different Wnt proteins, since the ability to stabilize β-catenin was not reported for Wnt-5a (36); rather, it might antagonize the function of other Wnts that stabilize β-catenin (37).

The target genes of Wnt signaling in stromal cells are still unclear. In the case of osteoclasts, slight changes of the expression of the RANKL and OPG/osteoclastogenesis inhibitor factor genes were observed. However, these changes do not fully explain the reduction of osteoclasts because the addition of an excess amount of RANKL to the cultures only partially rescued the osteoclastogenesis, suggesting that multiple genes are the targets of Wnt signaling in stromal cells. The following evidence suggests that changes of adhesive properties might be involved in the phenomena we observed. First, the hemopoietic cells were loosely attached to stromal cells in the cultures containing W3a-L-CM. Second, the colonies attached to stromal cells were small in size, but the number of CFCs was not affected in the cultures induced to express the stable form of β-catenin. However, further analyses are needed to clearly verify this hypothesis.

It had been reported that mouse hemopoietic tissues express Wnt-3a, -5a, and -10b. Interestingly, it is reported that Wnt-5a is expressed in both stromal cells and hemopoietic cells, while Wnt-3a and Wnt-10b are expressed specifically in the hemopoietic compartment (7, 9). This suggests that Wnt proteins secreted from hemopoietic cells bind frizzled receptors on stromal cells, transduce signals, and regulate the functions of the stromal cells. This possibility suggesting a reciprocal relationship between hemopoietic cells and stromal cells is very intriguing because such interactions may contribute to the homeostatic balance of hemopoietic cells.

The expression of other modulators of Wnt signaling, e.g., Frps (10), in hemopoietic tissue would be worth investigating. Also, some signals other than Wnt protein-elicited ones are known to up-regulate the level of β-catenin. For instance, integrins and growth factors stabilize β-catenin through integrin-linked kinase (38, 39). Indeed, ST2 cells express integrin-linked kinase (data not shown). These signaling pathways might also participate in the regulation of the level of β-catenin in stromal cells and might control hemopoiesis.

In conclusion, we showed in this study that stromal cells are a target of Wnt proteins, and that the production of hemopoietic lineages is regulated by Wnt signaling with selectivity and different sensitivities of the various hemopoietic lineages in the presence of stromal cells, i.e., under conditions reflecting the hemopoietic microenvironments in the body.

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