was terminated with the addition of 2 μl 10% SDS. The reaction mixtures were treated with 50 μg/ml proteinase K for 30 min at 37°C to digest the protein. Two microliters of loading buffer was added, and the DNA was extracted once with CIA (chloroform:isoamyl alcohol, 24:1). Samples were resolved by electrophoresis on a 1% agarose gel, after which the gel was stained with 0.5 μg/ml ethidium bromide in TAE buffer for 30 min. DNA bands were visualized by UV exposure and photographed on a UV transilluminator.

Western Blot

Western blot was performed as described with slight modifications (Woo et al., 2006). Briefly, cell lysates were prepared in RIPA buffer (25 mM HEPES [pH 7.8], 0.5 M NaCl, 5 mM EDTA, 1.5% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS, and 5 mM EDTA), supplemented with a protease inhibitor cocktail (Roche). Samples were subjected to SDS-PAGE and transferred to a PVDF membrane (Immobilon P, Millipore). Membranes were incubated with the indicated primary antibodies and horseradish peroxidase–labeled secondary antibodies and visualized by exposure to X-ray film using SuperSignal West Pico Chemiluminescence Substrate (Pierce).

BNS-22 Binding Assay

BNS-22–immobilized beads were prepared as described elsewhere (Kanoh et al., 2005; Kawatani et al., 2008). Purified human TOP2α (500 ng) was incubated with control or BNS-22 beads (20 μl) in the presence or absence of 100 μM BNS-22 or 100 μM ICRF-193 in binding buffer containing 40 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, 2 mM ATP, and 0.1% BSA in a total volume of 1 ml for 3 hr at 4°C. The reactant beads were washed with binding buffer without BSA, and the bound protein was eluted with SDS-PAGE sample buffer. The sample was resolved by SDS-PAGE and detected by western blot with anti-TOP2α.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.03.012.

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Cross-priming of $CD8^+$ T cells in vivo by dendritic cells pulsed with autologous apoptotic leukemic cells in immunotherapy for elderly patients with acute myeloid leukemia

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Objective. **The prognosis for elderly patients with acute myeloid leukemia (AML) remains dismal. To explore the potential of immunotherapy for improving clinical outcomes for these patients, we performed a phase I clinical trial of dendritic cell (DC)–based immunotherapy for elderly patients with AML.**

Materials and Methods. **Autologus monocytes were obtained after reducing tumor burden by chemotherapy. Immature DCs induced with granulocyte-macrophage colony-stimulating factor and interleukin-4 were pulsed with autologous apoptotic leukemic cells as antigens. DCs were administered intradermally to four patients five times at 2-week intervals. To facilitate DC migration to lymph nodes, injection sites were pretreated with killed *Streptococcus pyogenes* OK-432 one day before. DCs were coinjected with OK-432 to induce maturation and interleukin-12 production in vivo.**

Results. **Antileukemic responses were observed by an interferon-γ enzyme-linked immunospot assay or a tetramer assay in two of four patients. In a human leukocyte antigen–A*2402-positive patient, induction of $CD8^+$ T-cell responses to WT1- and human telomerase reverse transcriptase–derived peptides were observed, indicating cross-priming in vivo. The two patients with antileukemic immunity showed longer periods of disease stabilization than the other two patients.**

Conclusions. **This study demonstrates the immunogenicity of autologous DCs that cross-present leukemia-associated antigens from autologous apoptotic leukemic cells in vivo in elderly patients with AML.**

Management of elderly patients with acute myeloid leukemia (AML) remains a challenge because of a high rate of therapy-related mortality and chemotherapy resistance [1]. Antigen-specific immunotherapy, which is less toxic and kills leukemic cells through different mechanisms than chemotherapy, has the potential capacity to improve the clinical outcomes of these patients. Recent identification of several leukemia-associated antigens prompted us to develop immunotherapy for elderly patients with AML [2].

Active immunization by peptide vaccines can induce antileukemic immunity and clinical responses in AML [3–6]. Clinical trials of dendritic cell (DC)–based immunotherapy for AML have also been reported [7–12]. However, the trial using leukemic cell–derived DCs showed that the generation of leukemic cell–derived DCs was feasible in only a limited number of patients, and even in vaccinated patients the treatment could not induce clinical responses [9]. This may be due to lower immunostimulatory activity of leukemic cell–derived DCs than monocyte-derived DCs (MoDCs) [13]. Recently, the efficient generation of MoDCs from patients with AML has been demonstrated in vitro [14], providing a rationale for the use of MoDCs in immunotherapy for AML.

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There are several parameters to enhance the immunogenicity of MoDC vaccines. Whereas monocytes are cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 conventionally for 5 to 7 days to induce DCs, a shorter period of culture is sufficient to induce equivalently potent DCs [15]. Among DC maturation–inducing factors, microbial components that trigger the production of IL-12 are beneficial to induce effective adaptive immunity [16]. An extended period of stimulation with microbial components results in DC exhaustion in which DCs lose the capacity to produce IL-12 [17]. Thus, a short-term stimulation can generate optimal DCs that retain IL-12 production. Inflammation in the skin before DC injection facilitates DC migration to draining lymph nodes, leading to a stronger immune response [18,19]. Using apoptotic whole tumor cells as antigens may be instrumental in inducing multivalent immune responses [20].

We performed in vitro assays to optimize these parameters. Based on the results of these assays, we conducted a phase I clinical trial of immunotherapy for elderly patients with AML at the second or later remission setting, using DCs loaded with autologous apoptotic leukemic cells. The treatment was well-tolerated and safe and induced antileukemic immunity in two of four patients, which was associated with transient disease stabilization. Importantly, in one patient, cross-priming of leukemia antigen–specific $CD8^+$ T cells in vivo was explicitly demonstrated. This study indicates the safety and immunogenicity of immunotherapy using MoDCs that cross-present leukemic cell antigens in elderly patients with AML.

Materials and methods

Generation, maturation, and cryopreservation of DCs for in vitro assays

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers by density gradient centrifugation using Lympholyte H (Cedarlane, Ontario, Canada). Monocytes were purified using anti–CD14-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), or enriched by plastic adherence by incubating PBMCs at 37°C for 2 hours and removing nonadherent cells by pipetting. Monocytes were cultured with 800 IU/mL GM-CSF (Primmune, Kobe, Japan) and 500 IU/mL IL-4 (Primmune) in CellGro DC medium (CellGenix Technologie Transfer, Freiburg, Germany) for 3 days (3d-DCs) or 6 days (6d-DCs). In some experiments, 3d-DCs were frozen in CP-1 freezing medium (Kyokuto Pharmaceutical Industrial, Tokyo, Japan). CP-1 contains 12% hydroxymethyl starch and 10% dimethyl sulfoxide in normal saline and was mixed with 8% human serum albumin before use. DCs were matured with 0.1 KE/mL OK-432 (Picibanil; Chugai Pharmaceuticals, Tokyo, Japan), a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of *Streptococcus pyogenes* (group A) [21].

In vitro analysis of DC functions

Flow cytometric analysis, measurement of IL-12p70 production, T-cell–stimulatory capacity of DCs for allogeneic naive $CD4^+$ T cells, and the cytokine profile of $CD4^+$ T cells primed with DCs were analyzed as described previously [15,22].

Uptake of apoptotic cells by DCs and the cross-presenting capacity of DCs

Efficiency of uptake of apoptotic cells by DCs was assessed as described previously [23] using myeloid leukemia cell lines K562, OUN-1 [24] (Dr. Yasukawa, Ehime University, Japan), and a T-cell leukemia cell line MT2, which were killed by 120 Gy γ-irradiation and 48-hour serum-free culture in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan). To examine the cross-presenting capacity of DCs, human leukocyte antigen (HLA)-A*2402–positive, immature 3d-DCs were pulsed with HLA-A*2402–negative, Epstein-Barr virus–transformed lymphoblastoid cell lines, which were killed as described here. DCs were matured with OK-432 (0.1 KE/mL) and prostaglandin E_2 (1 μg/mL) (MP Biomedicals, Solon, OH, USA) for 6 hours, and cocultured with autologous T cells at a DC-to-T cell ratio of 1:10. IL-2 (50 IU/mL; Teceleukin; Shionogi & Co., Ltd., Osaka, Japan) was added on the next day. For a positive control, DCs pulsed with HLA-A*2402–restricted EBNA3B peptide (TYSAGIVQI; KURABO Industries, Osaka, Japan) were used. Expansion of EBNA3A- and EBNA3B-specific $CD8^+$ T cells were evaluated by HLA tetramer staining [25].

Clinical trial protocol

The protocol was approved by the Ethics Committee, Graduate School and Faculty of Medicine, Kyoto University. Each patient gave written informed consent in accordance with the Declaration of Helsinki. The primary and secondary objectives were the assessment of safety and immunological and clinical responses, respectively.

Autologous leukemic cells were harvested before induction chemotherapy. Patients were required to be between 16 and 79 years of age and have a diagnosis of AML according to World Health Organization criteria [26,27]. Patients were excluded if they had another concurrent malignancy, an active autoimmune disease, positivity for blood-borne infectious agents, or a history of penicillin allergy (because OK-432 contains penicillin). Patients were enrolled if 5×10^7 or more leukemic cells were harvested. Thereafter, patients were treated with chemotherapy. More than 4 weeks after the last chemotherapy, patients proceeded to the DC vaccination if leukemic cells in bone marrow (BM) were $<20\%$. In addition, to assess the clinical efficacy of DC vaccination, the presence of an evaluable lesion in BM, which was defined as 0.1% or more of leukemic cells by flow cytometry, was required. Furthermore, patients should have an Eastern Cooperative Oncology Group performance status of 0 to 2 and adequate vital organ functions. Patients were excluded if they had eligibility for hematopoietic stem cell transplantation or an uncontrollable infection. Concomitant chemotherapy and radiotherapy were prohibited.

DC vaccine generation

DC vaccines were generated from autologous monocytes under current Good Manufacturing Practice conditions. Autologous leukemic cells to be used as antigens were obtained as mononuclear cells (MNCs) by density gradient centrifugation over Ficoll-Hypaque (GE Healthcare, Buckinghamshire, UK) from BM and/or peripheral blood (PB) samples. MNCs were frozen in CP-1 freezing medium and stored at −150°C. Before added to DCs, MNCs were killed by 120 Gy

γ-irradiation and 48 hours serum starvation. Killing of MNCs was confirmed by the percentage of Annexin V–positive cells being 90% or more by flow cytometry and reduced uptake of [^{3}H]-thymidine to the baseline level.

Apheresis products, which were obtained with COBE Spectra (Caridian BCT, Lakewood, CO, USA) from 10 L blood, were processed by elutriation using Elutra (Caridian BCT) to enrich monocytes. At the time of apheresis, no leukemic cells were observed in the PB of the patients, as assessed by a routine clinical laboratory test. Monocytes were cultured with 800 U/mL GM-CSF and 500 U/mL IL-4 in CellGro DC medium in gas-permeable plastic bags (VueLife 118; CellGenix Technologie Transfer) at 37°C, 5% CO_2 to generate immature DCs. After 48 hours, DCs were pulsed with autologous apoptotic leukemic cells and 2 μg/mL keyhole-limpet hemocyanin (KLH; Biosyn Corporation, Carlsbad, CA, USA). The endotoxin level in the KLH preparation examined by the supplier was <0.1 IU/mg. After an additional 24 hours, DCs were frozen as immature DCs in CP-1 freezing medium and stored at −150°C.

Administration of the DC vaccine

A total of 1×10^7 DCs were intradermally injected at four sites in bilateral arms and thighs. Twenty-four hours before DC administration, the injection sites were pretreated by 0.2 KE/site OK-432. At the time of DC administration, DCs were thawed and mixed with 1 KE OK-432. Then, the mixture of DCs and OK-432 was injected. The DC administration was repeated at 2-week intervals for five administrations.

Monitoring of immunological and clinical responses

Antigen-specific immune responses were assessed at indicated time points. Immune responses to KLH and autologous leukemic cells were tested by skin delayed-type hypersensitivity tests and interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays. In addition, in a HLA-A*2402–positive patient, immune responses to HLA-A*2402–restricted peptides derived from leukemia-associated antigens were examined by IFN-γ ELISPOT assay and HLA tetramer staining. The peptides used in the assays were the natural $WT1_{235-243}$ peptide (CMTWNQMNL) [24], the modified $WT1_{235-243}$ peptide (CYTWNQMNL) [28], the human telomerase reverse transcriptase $(hTERT)_{461-469}$ peptide (VYGFVRACL) [29], and the lower matrix 65-kd phosphoprotein (pp65) of cytomegalovirus (CMV) (amino acids 328–336; QYDPVAALF) [30]. All peptides were purchased from Multiple Peptide Systems (San Diego, CA, USA). Both PBMCs and BM mononuclear cells (BMMCs) were subjected to assays before and after 1-week in vitro stimulation with antigen- or peptide-pulsed DCs in the presence of 15 U/mL IL-2 (Teceleukin). To evaluate clinical responses, percentages of leukemic cells in BM were monitored by morphology and flow cytometry at indicated time points.

Skin delayed-type hypersensitivity test

The 4×10^5 antigen-pulsed DCs were intradermally injected in the forearm. Sizes of induration and erythema were measured 48 hours later. Erythema that was 1.5-fold or larger in diameter than the antigen-unpulsed control was considered positive.

IFN-γ ELISPOT assay

IFN-γ ELISPOT assays (Mabtech, Nacka Strand, Sweden) were performed using antigen-pulsed DCs and peptide-pulsed C1R-A*2402 (Dr. Masafumi Takiguchi, Kumamoto University, Kumamoto, Japan). Stimulator cells were plated at 2×10^4 cells/well. As responder cells, fresh and in vitro–stimulated MNCs from PB and BM were plated with fresh MNCs at 1 to 2×10^5 cells/well and in vitro–stimulated MNCs at 1 to 2×10^4 cells/well. After overnight incubation, spots were developed using 3-amino-9-ethylcarbazole (Sigma Chemical, St Louis, MO, USA) and counted by KS ELISPOT compact (Carl Zeiss MicroImaging, Tokyo, Japan). Numbers of specific spot-forming cells were calculated by subtracting the number of spots with unpulsed DCs from the number of spots with antigen-pulsed DCs.

HLA tetramer staining

Natural $WT1_{235-243}$ peptide/HLA-A*2402 tetramer was purchased from Medical & Biological Laboratories (Nagoya, Japan). Modified $WT1_{235-243}$ peptide/HLA-A*2402 tetramer and a peptide derived from the HIV envelope (env) protein/HLA-A*2402 tetramer were produced as described previously [30]. Fresh and in vitro–stimulated MNCs were stained with a tetramer and fluorescein isothiocyanate–conjugated anti-CD8 monoclonal antibody (BD Biosciences) and analyzed by flow cytometery (FACSCalibur; BD Biosciences) [30].

Results

In vitro assays to optimize generation of DCs

To optimize generation of DCs, we performed in vitro functional assays. We first compared DCs differentiated from monocytes in the presence of GM-CSF and IL-4 for 3 days with 6-day differentiated DCs conventionally used in clinical trials. After 24-hour exposure to OK-432, both 3d-DCs and 6d-DCs showed similar levels of surface molecule expressions, IL-12p70 production, and T-cell stimulatory capacity for allogeneic naïve $CD4^+$ T cells (Supplementary Figure E1; online only, available at www.exphem.org), indicating that 3d-DCs have functions comparable with 6d-DCs. Next, we examined the capacity of 3d-DCs to cross-present apoptotic cell–associated antigens. At the DC-to-apoptotic cell ratio of 1:1, 11% to 33% of immature 3d-DCs incorporated apoptotic leukemia cell lines (Fig. 1A). Moreover, HLA-A*2402–positive DCs pulsed with killed lymphoblastoid cell lines from an HLA-A*2402–negative donor induced expansion of $CD8^+$ T cells specific for the HLA-A*2402–restricted epitopes of EBNA3A and EBNA3B (Fig. 1B), indicating the capacity of DCs to cross-present apoptotic cell–derived antigens.

An extended period of exposure of DCs to lipopolysaccharide leads to DC exhaustion [17], as indicated by loss of IL-12–producing capacity by DCs. To examine whether OK-432 induces DC exhaustion, we analyzed the maturation kinetics of OK-432–stimulated 3d-DCs. Upregulation of the surface molecules (Fig. 2A) and IL-12p70 production (Fig. 2B) became evident 4 and 8 hours after OK-432 stimulation, respectively. Maximal levels of surface molecule expressions and IL-12p70 production were observed at 48 hours. Next, we examined how many hours of exposure to OK-432 is sufficient to elicit a maturation signal to DCs, using 3d-DCs that were cultured for a total of 48 hours with different

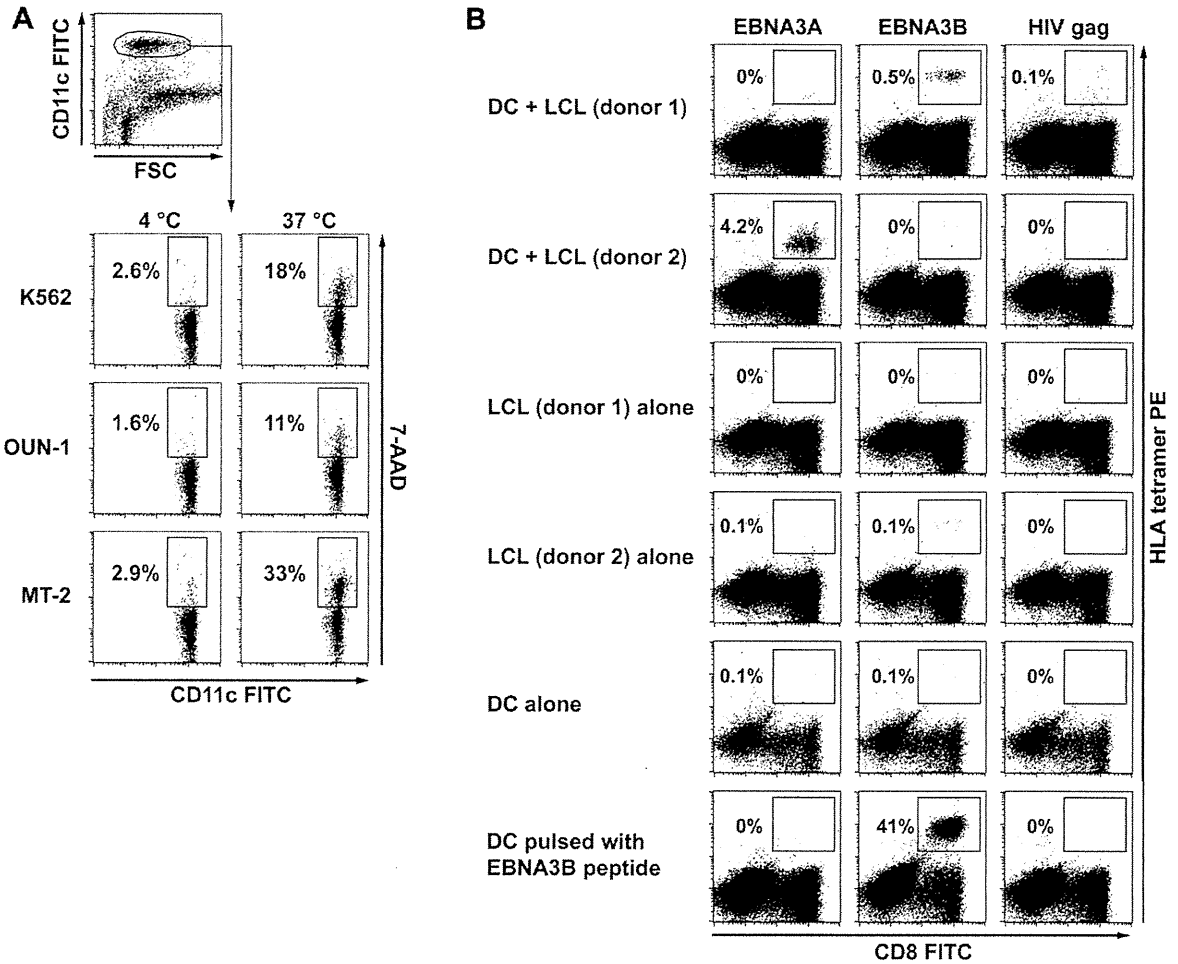

Figure 1. 3d-DCs incorporate apoptotic cells and cross-present cell-associated antigens. (**A**) Uptake of apoptotic cells by 3d-DCs. Apoptotic K562, OUN-1, and MT2 were labeled with 7-aminoactinomycin D (7-AAD) (20 μg/mL), and cocultured with immature 3d-DCs at a DC-to-apoptotic cell ratio of 1:1. After 4 hours of incubation at 4°C or 37°C, cells were stained with fluorescein isothiocyanate–conjugated anti-CD11c monoclonal antibody and analyzed by flow cytometry. Cells positive for both CD11c and 7-AAD were considered to be DCs that had phagocytosed apoptotic cells. (**B**) The cross-presenting capacity of DCs. Immature 3d-DCs from a HLA-A*2402–positive donor were pulsed with apoptotic HLA-A*2402–negative donor-derived lymphoblastoid cell lines (LCLs), matured with OK-432 and prostaglandin E_2, and cocultured with autologous T cells. For a positive control, DCs pulsed with the EBNA3B peptide were used as a stimulator. After 7 days, expansions of EBNA3A- and EBNA3B-specific $CD8^+$ T cells were evaluated by HLA tetramer staining. Dead cells are excluded by staining with propidium iodide. Numbers shown indicate percentages of tetramer-positive cells among $CD8^+$ cells. Representative data from two experiments are shown.

durations of exposure to OK-432 at the start of culture. As short as 2-hour exposure upregulated CD83 and CD86 (Fig. 2C) and induced IL-12p70 production (Fig. 2D) during the subsequent 46-hour culture without OK-432. Although at the time of 8-hour exposure, the induction of CD83, CD86 (Fig. 2A), and IL-12p70 (Fig. 2B) was low, 8-hour exposure was sufficient to induce maximal levels of CD83 and CD86 expression (Fig. 2C) and IL-12p70 production (Fig. 2D). Notably, although initial 24-hour exposure to OK-432 induced the maximal levels of CD83 and CD86 expression (Fig. 2C), DCs did not produce a detectable level of IL-12p70 during the last 24-hour culture (Fig. 2D). These data indicate that, like lipopolysaccharide [17], OK-432–induced IL-12p70 production was limited within the first 24 hours and most active between 8 and 24 hours after OK-432 stimulation. The functional significance of ongoing IL-12p70 production by DCs in priming naïve $CD4^+$ T cells was supported by the data that 3d-DCs matured with OK-432 for 6 hours showed a superior capacity to induce IFN-γ–producing T cells to those matured for 24 hours (Fig. 2E). Thus, extended stimulation with OK-432 induces DC exhaustion. To avoid it, we decided to administer immature DCs together with OK-432 to patients and to induce DC maturation in vivo.

It is convenient to prepare a large number of DCs from a single batch of apheresis and freeze them in aliquots. We assessed the effect of cryopreservation on DCs. Whereas cryopreserved immature 3d-DCs showed somewhat higher percentages of dead cells after 24-hour culture with or

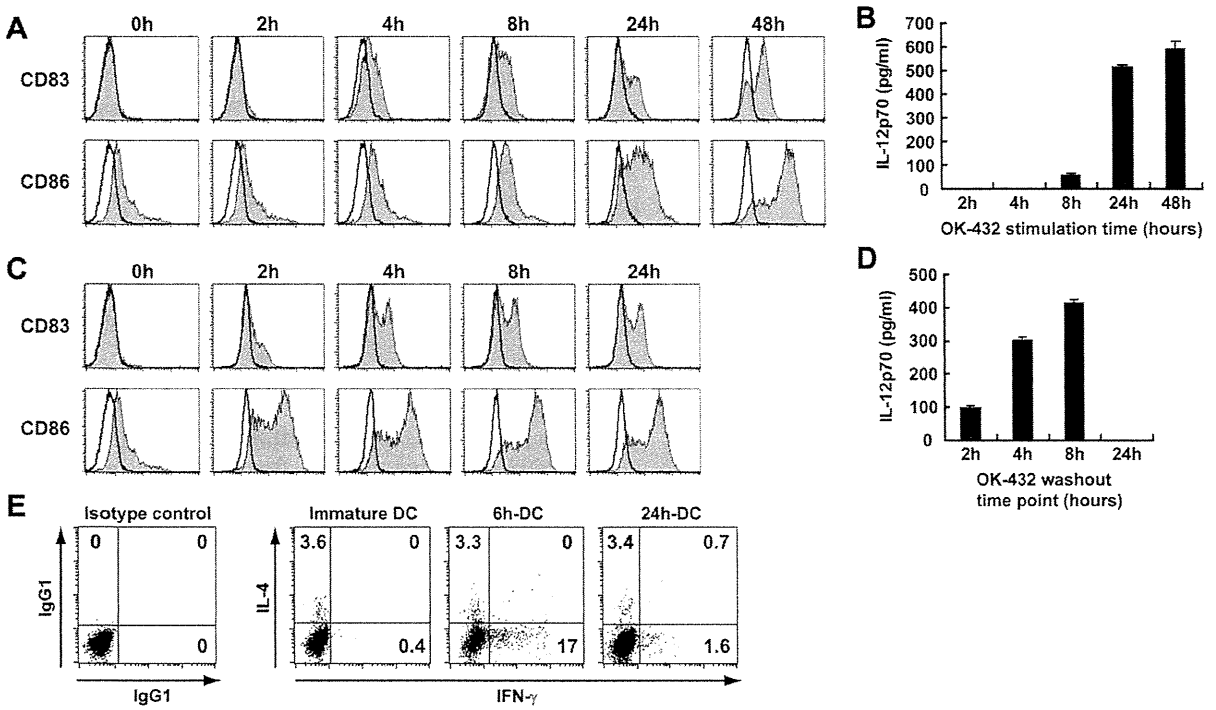

Figure 2. Short-term stimulation with OK-432 is optimal to generate Th1-inducing mature DCs. (**A**, **B**) Immature 3d-DCs were cultured in the presence of OK-432 (0.1 KE/mL) for indicated time periods, then harvested and analyzed. (**C**, **D**) Immature 3d-DCs were cultured in the presence of OK-432 for indicated time periods, washed, replated, and further cultured for a total of 48 hours. Cells and supernatants harvested at 48 hours were analyzed. (**A**, **C**) Expression of CD83 and CD86 was analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms indicate staining with isotype controls. (**B**, **D**) IL-12p70 production in culture supernatants of DCs (5×10^5 cells/mL) were measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. (**E**) Naïve $CD4^+$ T cell differentiation induced by DCs. Immature 3d-DCs were matured with OK-432 (0.1 KE/mL) for 6 or 24 hours and cocultured with allogeneic naïve $CD4^+$ T cells for 7 days. Cytokine profiles of T cells were analyzed by intracellular cytokine staining. Numbers indicate percentages of cells in each quadrant. Representative data from four experiments are shown.

without OK-432, and tended to produce a lower amount of IL-12p70 upon OK-432 stimulation as compared with non-cryopreserved DCs (Supplementary Figure E2A, C; online only, available at www.exphem.org), similar levels of CD83 and CD86 expression were induced by OK-432 in both DCs (Supplementary Figure E2B; online only, available at www.exphem.org). Thus, although cryopreservation of immature DCs impaired their function to some extent, cryopreserved DCs largely retained the viability and expression of immunostimulatory molecules. Considering the practical convenience to prepare a stock of DCs at one time, we decided to freeze DCs as immature DCs. Taken together, these data demonstrate that DCs generated in the present study are capable of inducing $CD8^+$ T-cell responses to apoptotic cell–derived antigens, and that immature DCs can be cryopreserved without critical loss of functions.

Patients, feasibility, and safety

Thirteen patients were recruited to the study for the leukemic-cell harvest at the onset of AML. After chemotherapy, four patients were eligible for DC vaccination (Table 1). In these patients, $>5 \times 10^7$ DCs for five vaccinations could be generated from a single apheresis. Autologous apoptotic leukemic cells were added to DCs as antigens at leukemic cell-to-DC ratios of 1:3.3 to 1:6.5, depending on the numbers of collected leukemic cells (Supplementary Table E1; online only, available at www.exphem.org). Status of PB and BM at the time of apheresis are shown in Supplementary Table E1 (online only, available at www.exphem.org). Representative data of surface molecule expressions on DCs are shown in Supplementary Figure E3 (online only, available at www.exphem.org).

All of the patients completed the five vaccinations safely (Table 1). In all the patients, grade 1 to 2 fever and grade 2 skin reactions at the injection sites were observed. The fever was resolved within 2 days after vaccination and most likely related to administration of OK-432. The skin reactions at the injection sites were transient and characterized by erythema, pruritus, and tenderness. No significant toxicities to vital organs or signs of autoimmunity were observed.

Induction of antigen-specific immune responses to KLH and leukemic cells

Induction of an immune response to KLH was detected by skin delayed-type hypersensitivity tests and/or IFN-γ ELISPOT assays in three patients, with the exception of patient no. 4

Table 1. Patient characteristics and results of the DC vaccination

Patient no.	Age/Sex	Diagnosis	DC vaccination was started		LC in BM at the first vaccination[a] (%)	Adverse effects[b]	Immune response		Clinical response	Died at (days after the last vaccination)
			After the last CT (d)	After diagnosis (d)			KLH	LC		
1	76/F	AML-MRC	82	93	1.8	Fever (1)	Yes	No	PD	186
						Injection site reaction (2)			Died of sepsis with leukemia	
2	75/M	AML-MRC	40	155	0.6	Fever (1)	Yes	Yes	Transient disease stabilization	391
						Injection site reaction (2)			Died of leukemia	
3	70/M	AML-MRC	44	344	2.9	Fever (2)	Yes	Yes	Transient disease stabilization	192
						Injection site reaction (2)			Died of sepsis with leukemia	
4	66/M	AML M2	67	144	0.2	Fever (1)	No	No	PD	66
						Injection site reaction (2)			Died of leukemia	

AML-MRC = acute myeloid leukemia with myelodysplasia-related changes; CT = chemotherapy; F = female; LC = leukemic cells; M = male; PD = progressive disease.
[a]Percentages of leukemic cells in bone marrow were determined by flow cytometry.
[b]Numbers in parentheses indicate grade of toxicity according to the National Cancer Institute-Common Terminology Criteria for Adverse Events version 3.0.

(Table 1 and data not shown). Two patients (patient nos. 2 and 3) showed induction of immune responses to leukemia-associated antigens. In patient no. 2, who was HLA-A*2402–negative, IFN-γ ELISPOT assays using autologous leukemic cell–pulsed DCs revealed the induction of antileukemic immunity in PBMCs and BMMCs without in vitro stimulation after the fourth vaccination (Fig. 3A). The antileukemic immune response was still detected 1 month after the fifth vaccination in in vitro–stimulated PBMCs and BMMCs (Fig. 3B), but was no longer detected without in vitro stimulation (Fig. 3A). We could not test antileukemic immunity at subsequent time points in this patient because the patient developed leukocytopenia, probably owing to progression of myelodysplastic syndrome.

In patient no. 3, who was HLA-A*2402–positive, HLA-A*2402–restricted peptides from WT1 and hTERT were used in immunological monitoring. $CMVpp65_{328-336}$ peptide was used as a positive control in ELISPOT assays (Fig. 4B). No responses to the leukemia-associated antigens were observed until the fourth vaccination. However, 2 months after the fifth vaccination, positive responses to the modified $WT1_{235-243}$ and the $hTERT_{461-469}$ peptides were detected in in vitro–stimulated PBMCs by HLA tetramer staining (Fig. 4A) and an IFN-γ ELISPOT assay (Fig. 4B), respectively. The PBMCs binding to the modified $WT1_{235-243}$ peptide/HLA-A*2402 tetramer also bound to the natural $WT1_{235-243}$ peptide/HLA-A*2402 tetramer (Fig. 4A), indicating that these cells were capable of recognizing the natural WT1 peptide presented on leukemic cells. These responses were short-lived and almost completely disappeared 3 months after the fifth vaccination. No responses were detected in PBMCs or BMMCs without in vitro stimulation (data not shown). Thus, the vaccinations induced HLA class I–restricted, antileukemic immunity, indicating that the DCs cross-presented leukemia-associated antigens in vivo. In addition, in patient no. 2, leukemic cell-reactive T cells were detected in BM (Fig. 3), the main tumor site in leukemia.

Clinical outcomes

The two patients with antileukemic immunity had longer periods of disease stabilization than the other two patients without antileukemic immunity (Fig. 5A). Notably, in patient no. 3, the percentages of leukemic cells in BM dropped from 11% to 5.2% during the second month after the fifth vaccination, when a positive antileukemic immunity was observed (Fig. 5B). Thus, these observations suggest that induction of antileukemic immunity was associated with extended the periods of disease stabilization in these patients.

Discussion

Novel therapies with less toxicity are necessary for intractable AML in elderly patients. In this study, we conducted a phase I clinical trial of immunotherapy for such patients using DCs pulsed with autologous apoptotic leukemic cells.

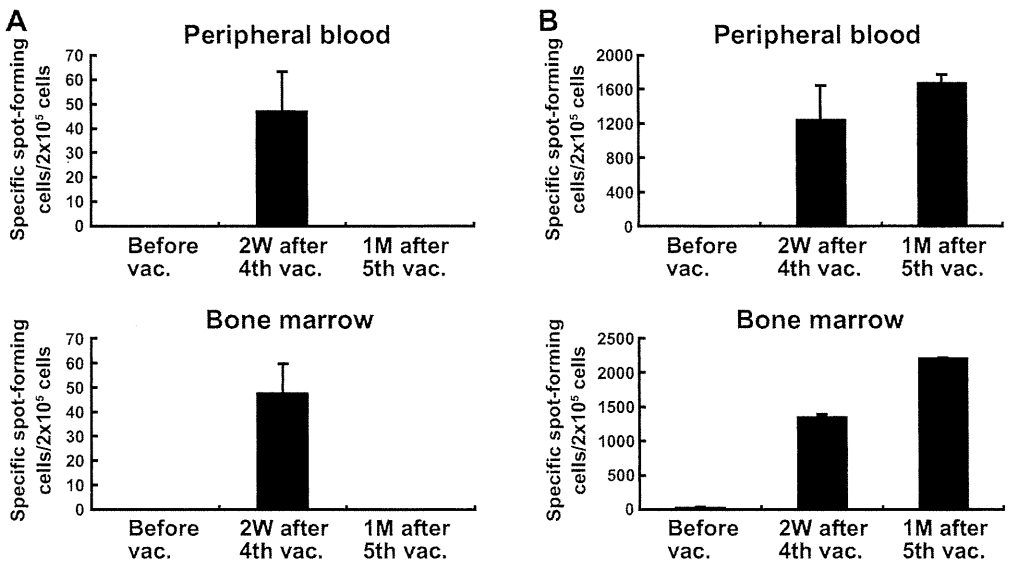

Figure 3. IFN-γ ELISPOT assay in patient no. 2. MNCs from PB and BM were obtained at indicated time points and subjected to IFN-γ ELISPOT assays directly after isolation (**A**) or after 1 week of stimulation with antigen-pulsed DCs (**B**). In IFN-γ ELISPOT assays, 2×10^5 MNCs (**A**) and 1×10^4 MNCs (**B**) were incubated with 1×10^4 leukemic cell–pulsed or unpulsed DCs. Numbers of specific spot-forming cells per 2×10^5 MNCs, calculated by subtracting numbers of spots with unpulsed DCs from numbers of spots with leukemic cell–pulsed DCs. Error bars indicate the standard deviation of duplicate measurements.

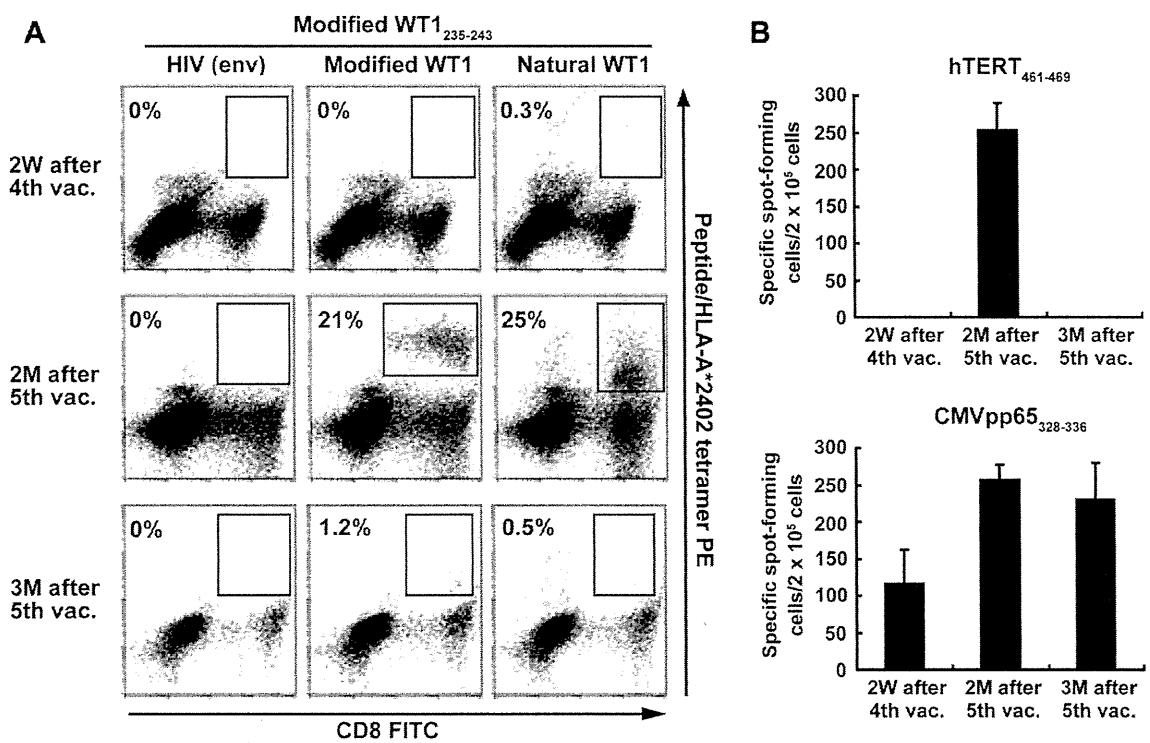

Figure 4. Immune responses in patient no. 3. (**A**) HLA tetramer staining. MNCs from PB were obtained at indicated time points, stimulated for 1 week with DCs pulsed with the modified $WT1_{235-243}$ peptide, stained with phycoerythrin-labeled peptide/HLA-A*2402 tetramers and fluorescein isothiocyanate–labeled anti-CD8 monoclonal antibody, and analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Numbers indicate percentages of tetramer-positive cells among $CD8^+$ cells. (**B**) IFN-γ ELISPOT assay. MNCs were stimulated for 1 week with DCs pulsed with the $hTERT_{461-469}$ or $CMVpp65_{328-336}$ peptide, and subjected to IFN-γ ELISPOT assays. In the assays, 2×10^4 MNCs were incubated with 2×10^4 C1R-A*2402 pulsed with or without the $hTERT_{461-469}$ or $CMVpp65_{328-336}$ peptide. Before vaccination, the assay was performed using DCs as a stimulator, which induced many nonspecific spots. Thus, the data before vaccination are not shown. Numbers of specific spot-forming cells per 2×10^5 MNCs, calculated by subtracting numbers of spots with unpulsed C1R-A*2402 from numbers of spots with antigen-pulsed C1R-A*2402, were depicted. Error bars indicate the standard deviation of duplicate measurements.

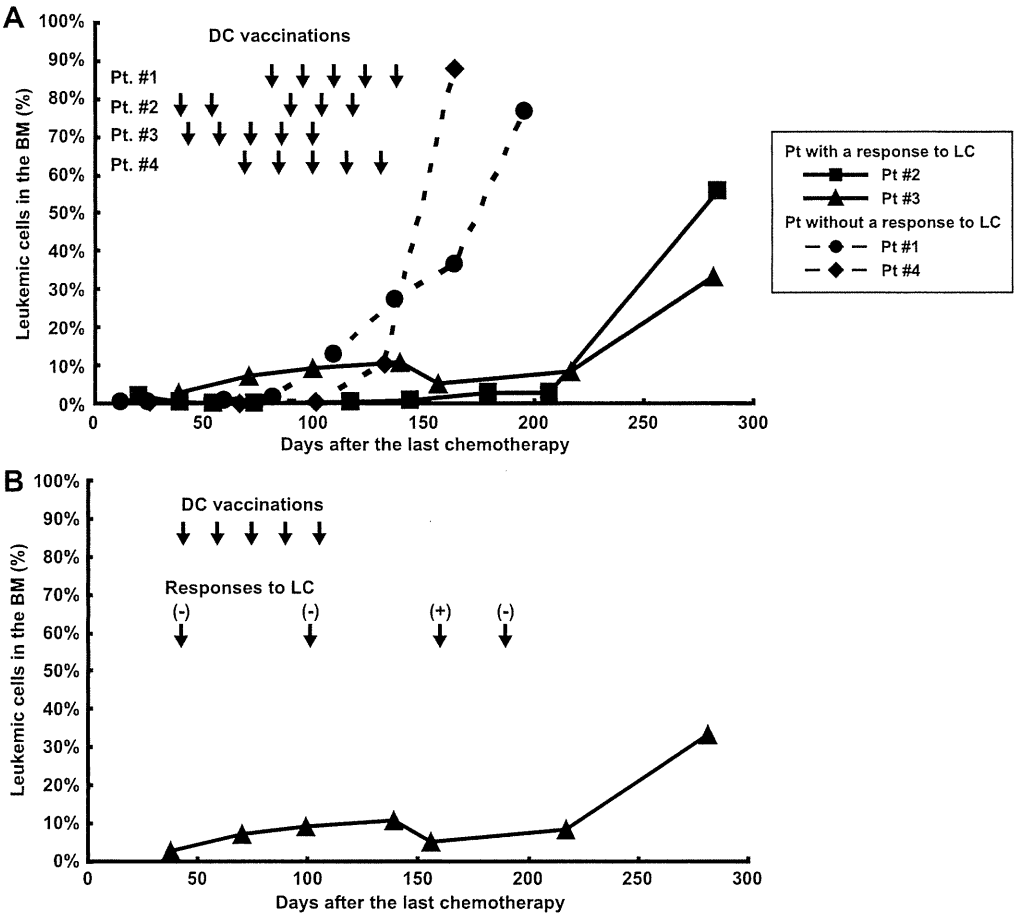

Figure 5. Clinical courses during the DC vaccination. (**A**) Percentages of leukemic cells in BM as determined by flow cytometry in four vaccinated patients are shown. Solid lines indicate patients with immune responses to leukemic cells (LCs) (patients 2 [■] and 3 [▲]). Dashed lines indicate patients without immune responses to LCs (patients 1 [●] and 4 [◆]). Arrows indicate time points when DC vaccines were administered to each patient. (**B**) The clinical course of patient no. 3. Arrows indicate time points when immunological monitoring was performed. Plus (+) or minus (−) signs indicates that immune responses to leukemic cells were detected or not detected at that time point, respectively.

Induction of antileukemic immunity was observed in two of four vaccinated patients. This is the first study that demonstrates cross-priming of $CD8^+$ T cells by DCs pulsed with apoptotic leukemic cells in vivo in humans, thus providing a proof of principle of this approach. The limited number of patients prevented us from drawing any definitive conclusion regarding clinical efficacy from the present trial. However, longer periods of disease stabilization observed in the two patients with antileukemic immunity compared to the other two patients without antileukemic immunity implied that induction of antileukemic immunity might have impacted on the clinical course of these patients.

There are several features in the method of DC vaccination in this trial: short-term 3-day culture to generate DCs in an attempt to reduce labor, cost, and time; use of whole leukemic cells as antigens to induce multivalent immune responses; use of the microbial adjuvant OK-432 as a maturation-inducing factor to generate Th1-inducing DCs; in vivo maturation of DCs to avoid DC exhaustion by extended stimulation in vitro with OK-432; and prior induction of inflammation at the injection sites to facilitate DC migration to draining lymph nodes.

We used autologous apoptotic leukemic cells as antigens because several studies have shown that apoptotic cells are more efficiently cross-presented by DCs to $CD8^+$ T cells than soluble antigens such as tumor lysate [31–34]. Furthermore, MoDCs has been shown to cross-present apoptotic leukemic cells to $CD8^+$ T cells in vitro [35]. Apoptotic cells as antigens also have advantages over peptides, in that the DCs have the ability to process multiple antigens from the apoptotic cells and present those antigens on their own HLA molecules. In this study, we clearly showed that MoDCs cross-presented leukemia-associated antigens WT1 and hTERT from apoptotic leukemic cells. Furthermore, T cells reactive to leukemic cells were detected in BM.

A murine study has shown that DC maturation not by inflammatory cytokines but by pathogen-derived components is crucial for DCs to acquire the capacity to differentiate naïve $CD4^+$ T cells into effector T cells [16]. We used OK-432, a preparation of killed *Streptococcus pyogenes* [21], which strongly triggers DC maturation through Toll-like receptor 4 [36–39]. We showed that, like lipopolysaccharide [17], longer stimulation with OK-432 induces DC exhaustion, resulting in the reduced capacity of DCs to induce Th1 responses. Several preclinical studies have shown that DCs briefly exposed to Toll-like receptor ligands are better inducers of Th1-type and cytotoxic T-cell responses [17,40,41]. Moreover, a clinical trial suggests superiority of briefly matured DCs in pediatric patients with cancer [42]. In this trial, we administered immature DCs together with OK-432 to avoid DC exhaustion before administration. The induction of IFN-γ detected by the ELISPOT assay implied IL-12 production by DCs in vivo.

Only a small proportion of intradermally administered DCs reach draining lymph nodes [43,44]. In a mouse model, pretreatment of administration sites with inflammatory cytokines enhance DC migration to regional lymph nodes [18]. Based on this finding, we pretreated administration sites with a low dose of OK-432. Because of unavailability of a cell-processing facility for cells labeled with indium-111 oxyquinoline [43,44], we could not evaluate the efficiency of DC migration to lymph nodes. Whether this administration procedure is superior to others should be evaluated in future studies.

In this study, multiple vaccinations were required to elicit antileukemic immunity, which rapidly declined after cessation of vaccination. Maintenance of antileukemic immunity might lead to improvement of clinical efficacy, and might be fulfilled by increasing the number of vaccinations, which was, however, impossible in this study because of the limited availability of autologous leukemic cells. Thus, if a peptide is available for the induced antileukemic $CD8^+$ T-cell response, peptide vaccination may be added after DC vaccination. Furthermore, blockade of immunosuppressive mechanisms may be combined.

In conclusion, we demonstrated the feasibility, safety, and immunogenicity of DC-based immunotherapy for elderly patients with AML. Cross-priming of $CD8^+$ T cells by DCs pulsed with autologous apoptotic leukemic cells was provoked in vivo. The results were promising, yet further intensification of vaccine potency is clearly required. This novel therapeutic approach may lead to improvement of clinical outcomes in elderly patients with AML, which has been difficult to achieve with other therapeutic approaches.

Acknowledgments

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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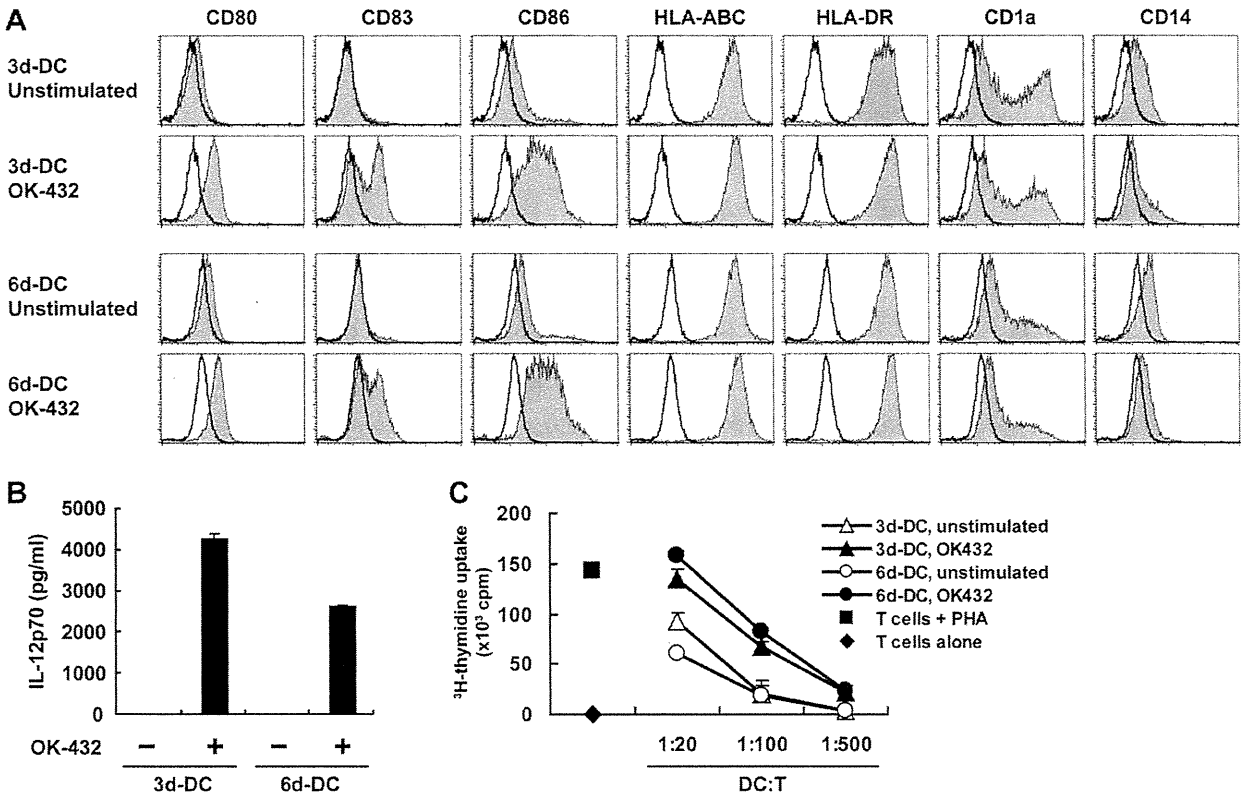
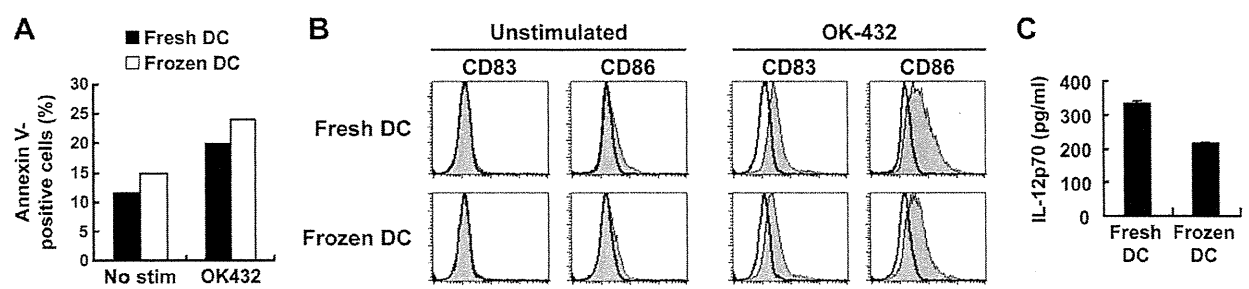
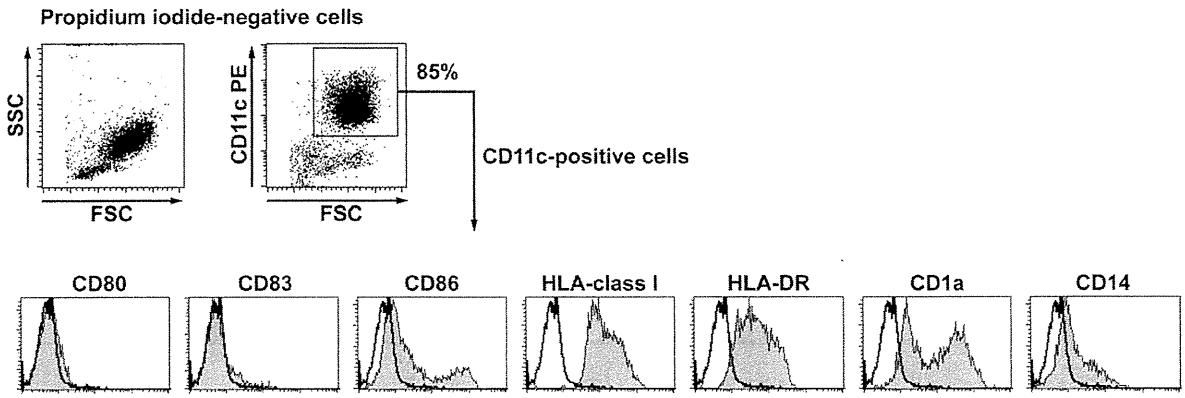


Supplementary Figure E1. 3d-DCs and 6d-DCs have comparable T-cell stimulatory capacity. (**A**) Expressions of surface molecules on DCs. Unstimulated or OK-432-stimulated DCs were analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms indicate staining with isotype controls. (**B**) IL-12p70 production by DCs (5×10^5 cells/mL) stimulated with OK-432 (0.1 KE/mL) for 24 hours was measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. (**C**) Proliferation of naive $CD4^+$ T cells stimulated with DCs. Allogeneic naive $CD4^+$ T cells were cocultured with DCs at indicated DC to T-cell ratios. On day 4, 1 Ci of [^{3}H]-thymidine was added. After 16 hours of further incubation, thymidine uptake was counted. Naive $CD4^+$ T cells were stimulated with 10 μg/mL phytohemagglutinin as a positive control. Representative data from three experiments are shown.



Supplementary Figure E2. Effects of cryopreservation on immature 3d-DCs. (**A**) Viability of fresh and frozen 3d-DCs after 24 hours of incubation with or without OK-432 (0.1 KE/mL) were evaluated by staining with Annexin-V. Percentages of Annexin-Vpositive cells are indicated. (**B**) Expression of surface molecules on fresh and frozen DCs after 24 hours of incubation with or without OK-432. (**C**) IL-12p70 production by fresh and frozen DCs (5×10^5 cells/mL) induced by 24-hour stimulation with OK-432 was measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. Representative data from four experiments are shown.



Supplementary Figure E3. Expression of surface molecules on DCs for vaccination. Cryopreserved DCs from patients were thawed, stained, and analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Numbers indicate percentages of cells in each quadrant. Representative data from patient no. 1 are shown.

Supplementary Table E1. DC vaccine generation

	At the time of apheresis				
Patient no.	Days after the last CT	PB WBC (/L)	PB Mo (%)	BM LC[a] (%)	Antigen dose (LC:DC)
1	74	4700	7	0.9	1:5
2	31	3000	9	2.0	1:6.5
3	43	3900	15	0[b]	1:6
4	46	4800	16	0.3	1:3.3

CT = chemotherapy; LC = leukemic cells; Mo = monocytes.
[a]Percentages of leukemic cells in bone marrow were determined by flow cytometry.
[b]Patient 3 was in complete remission at the time of apheresis. The patient subsequently relapsed and became eligible for DC vaccination.

Expert Opinion

Targeting the Wnt/β-catenin signaling pathway in human cancers

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Introduction: The Wnt/β-catenin signaling pathway plays a pivotal role in the regulation of cell growth, cell development and the differentiation of normal stem cells. Constitutive activation of the Wnt/β-catenin signaling pathway is found in many human cancers, and is thus an attractive target for anti-cancer therapy. Specific inhibitors of this pathway have been keenly researched and developed.

Areas covered: This review discusses the potential of inhibiting the Wnt/β-catenin signaling pathway, as a therapeutic approach for cancer, along with an overview of the development of specific inhibitors.

Expert opinion: Cancer stem cells (CSCs) play a significant role in the development and recurrence of several cancers, and Wnt/β-catenin signaling is important for the proliferation of CSCs. Inhibition of Wnt/β-catenin signaling is therefore a promising treatment approach. Progress has been made in the development of screening methods to identify Wnt/β-catenin signaling inhibitors. Biomarker-based screening is an effective and promising method for the identification of compounds of interest.

Keywords: cancer, cancer stem cell, high-throughput screening, Wnt, β-catenin

Expert Opin. Ther. Targets (2011) 15(7):873-887

1. Introduction

Wnt signaling pathways are important in developmental processes, cell growth and differentiation, and they have been well studied in relation to their role in developmental events [1-4]. Wnt signaling pathways can be divided into three main branches: the canonical Wnt/β-catenin pathway, the non-canonical planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway [1,4]. This review focuses on the Wnt/β-catenin signaling pathway. The control of the Wnt/β-catenin signaling pathway occurs through a variety of mechanisms aimed at preventing its aberrant activation. This signaling pathway has been associated with various type of cancer. The dysregulation of the Wnt/β-catenin signaling pathway was first investigated in a report linking alteration in Wnt/β-catenin signaling to the progression of colon cancer [5,6]. The findings of this study led to the development of molecular targeted therapy using inhibitors of the Wnt pathway. Antibody and siRNA approaches to the inhibition of the Wnt pathway have shown considerable efficacy [7-11], and conventional agents have been reported to contain inhibitors of this signaling pathway [12-16]. To identify small-molecule inhibitors of the Wnt pathway, several different screening techniques are used. In the present work, the potential of Wnt/β-catenin signaling pathway inhibitors as anti-cancer therapies is discussed.

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Article highlights.

- Aberrant activation of the Wnt/β-catenin signaling pathway is associated with various human cancers.
- Wnt/β-catenin signaling activity defines the characteristics of cancer stem cells.
- Inhibition of Wnt/β-catenin signaling is a potent strategy for cancer therapy.
- Progress has been made in the development of screening methods to identify Wnt/β-catenin signaling inhibitors.
- Biomarker-based screening is an effective and promising method for the identification of compounds of interest.

This box summarizes key points contained in the article.

2. Overview of the canonical Wnt/β-catenin signaling pathway

The name Wnt is derived from the Drosophilia segment polarity gene Wingless and mouse proto-oncogene Int-1. Wnt ligands are secreted glycoproteins that have been reported to have 19 isoforms in humans [17], and they bind to the Frizzled (Fz) receptor and the low density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor on the cell membrane, which initiates Wnt signaling. Wnt signaling activity can be downregulated by Wnt inhibitory factor-1 (WIF-1) and soluble frizzled-related proteins (SFRP), which inhibit Wnt signaling by directly binding to Wnt ligands [18,19], and Dickkopf (Dkk) proteins, which act by directly binding to LRP5/6 [20].

In the absence of Wnt signals, adenomatous polyposis coli (APC), Axin, glycogen synthase kinase-3β (GSK3β) and casein kinase 1α (CK1α) form a complex called the 'β-catenin destruction complex'. GSK3β and CK1α target serine/threonine residues at the N terminus of β-catenin for phosphorylation [21,22]. Phosphorylated β-catenin is recognized and polyubiquitinated by β-transducin-repeat-containing protein (β-TrCP), a component of a ubiquitin ligase complex [23,24], targeting β-catenin for degradation by the 26S proteasome (Figure 1).

On the other hand, the binding of Wnt ligands to Fz receptors and LRP5/6 coreceptors induces the phosphorylation of Disheveled (Dvl) and prevents GSK3β-dependent phosphorylation of β-catenin. Stabilized β-catenin translocates into the nucleus and interacts with T-cell factor (TCF)/lymphocyte enhancer factor (LEF). In the absence of β-catenin, TCF/LEF, which interacts with Groucho and HDAC, acts as a repressor of the transcription [25,26]. β-catenin displaces Groucho and HDAC from TCF/LEF, and the β-catenin-TCF/LEF complex activates the transcription of target genes. The β-catenin/TCF complex regulates the transcription of a number of genes associated with cell proliferation and apoptosis, as well as the expression of growth factors. Typical β-catenin/TCF target genes that are associated with cell proliferation are c-myc and cyclin D1. The c-myc oncogene regulates cell cycle progression and apoptosis. Cyclin D1 activates cyclin-dependent kinases leading to cell cycle progression. These genes are overexpressed in many human cancers including colorectal cancer [27]. The expression of c-jun and fos-related antigen 1 (fra-1), which are components of the activator protein 1 (AP-1) transcription complex, are also upregulated by Wnt/β-catenin signaling through a TCF-binding site in the promoter. In addition, the expression of the anti-apoptotic protein survivin is induced by β-catenin/TCF-mediated transcription [28]. VEGF plays a critical role in the proliferation of blood vessels and is associated with cancer progression [29]. Because VEGF is a target of the β-catenin/TCF complex [30], activation of the Wnt/β-catenin signaling pathway promotes the invasion and metastasis of cancer cells. In addition to the Wnt target genes mentioned above, an increasing number of Wnt target genes have been reported (see The Wnt homepage: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/). Transcriptional regulation mediated by the β-catenin/TCF complex requires a number of co-activators, including histone acetyltransferase p300 and cAMP ressponse element binding protein (CREB)-binding protein (CBP), which interact with the C-terminus of β-catenin to activate β-catenin/TCF-mediated transcription [31], and B-cell chronic lymphocytic leukemia/lymphoma 9 protein (Bcl-9) and Pygopus, which act as co-activators by interacting with the N-terminus of β-catenin [32,33].

3. Wnt signaling in cancers

Aberrant activation of Wnt/β-catenin signaling is observed in many human cancers. Genetic mutations of Wnt signaling pathway components are primarily responsible for this aberrant activation. Genetic mutations of the components of the β-catenin destruction complex are common in human cancers (Table 1). Among them, mutations in the APC gene have been well studied for their role in the dysregulation of Wnt/β-catenin signaling. APC mutations were first found as the cause of familial adenomatous polyposis (FAP) [2]. FAP is an inherited disease characterized by the early onset of hundreds to thousands of colorectal adenomas. If the colon is not removed, FAP patients are at a risk of developing colorectal cancer. Mutations in β-catenin itself are also found in colorectal cancer. Approximately 80 – 90% of sporadic colorectal cancers harbor mutations in the APC gene, whereas β-catenin mutations are detected in 50% of cases without APC mutations, representing less than 10% of all colorectal cancers [34,35]. These mutations cause β-catenin to escape the degradation process and lead to nuclear stabilized β-catenin accumulation. Similarly, overexpression of Wnt ligands or downregulation of Wnt antagonists (Dkk, SFRP and WIF) have been reported in several human cancers. The stimulation of Wnt ligands stabilizes β-catenin and drives the transcription of target genes.

Resistance to chemotherapy and minimal residual disease (MRD) are obstacles to improving survival rates. Cancer stem cells (CSCs) are similar to normal stem cells in many

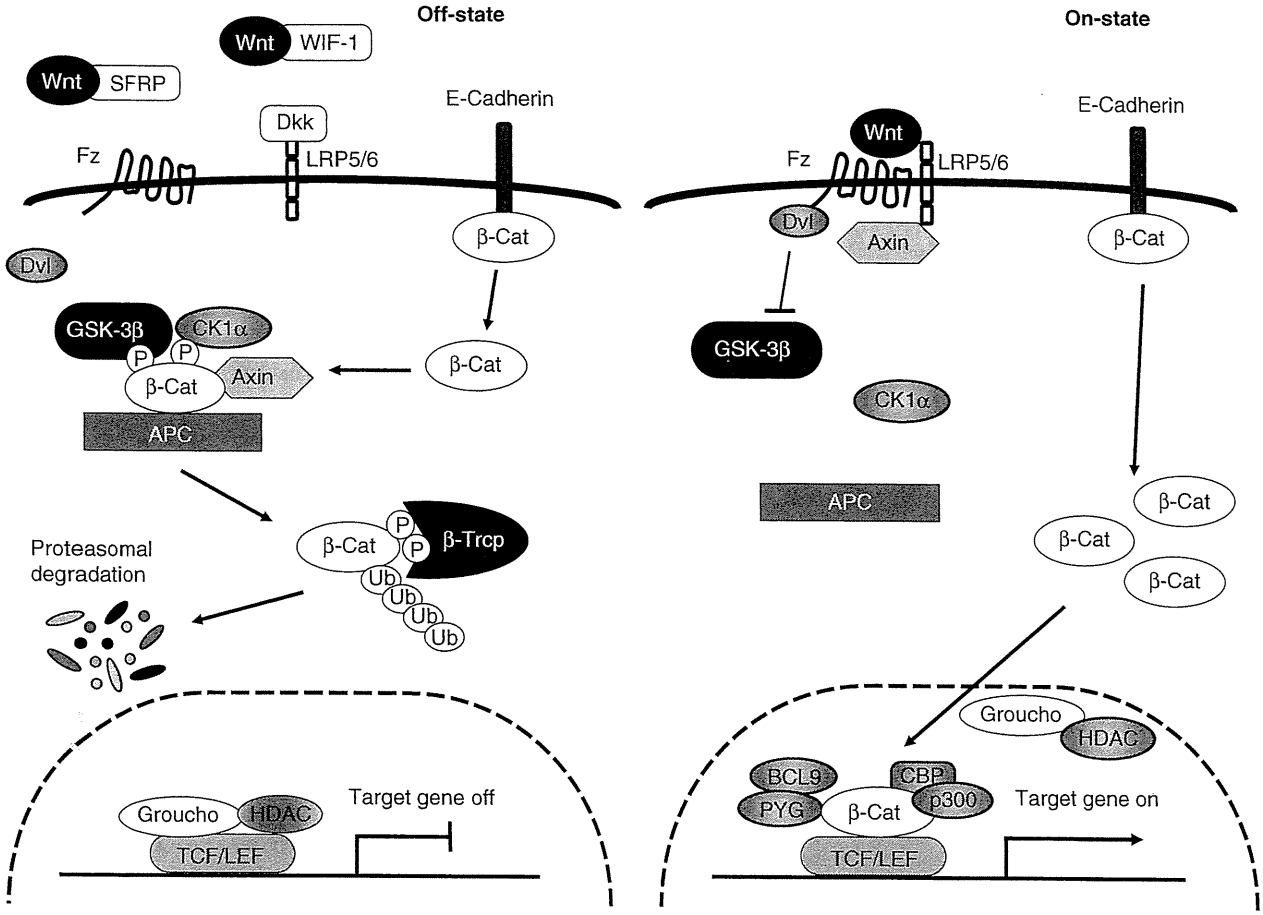


Figure 1. The Wnt/β-catenin signaling pathway. The majority of β-catenin (β-Cat) is localized to the cell membrane, where it binds to the cytoplasmic domain of E-cadherin, a cell adhesion molecule. A small part of free β-catenin exists in the cytoplasmic pool. Soluble frizzled-related proteins (SFRP) and Wnt inhibitory factor-1 (WIF-1) inhibit binding of Wnt ligands to frizzled (Fz) receptors. Dickkopf (Dkk) interacts with low density lipoprotein receptor-related protein 5/6 (LRP5/6) to inhibit binding of Wnt ligands. In the absence of Wnt/β-catenin signaling, "Wnt off" state (Left panel), free cytoplasmic β-catenin is phosphorylated by a complex formed by casein kinase 1α (CK1α), glycogen synthase kinase-3β (GSK3β), adenomatous polyposis coli (APC), and Axin. Phosphorylated β-catenin is recognized by β-transducin repeat-containing protein (β-TrCP) and subsequently ubiquitinated, which targets it for degradation by the proteasome. Interactions between the Wnt ligand and the Fz/LRP receptor trigger Wnt signal transduction, as seen in the 'Wnt on' state (Right panel). Phosphorylation of β-catenin is suppressed and β-catenin escapes from the degradation. Free cytoplasmic β-catenin translocates to the nucleus and forms a complex with TCF/LEF. The β-catenin-TCF complex activates the transcription of target genes, such as cyclin D1 and c-myc.

Bcl-9: B-cell chronic lymphocytic leukemia/lymphoma 9 protein; CBP: cAMP response element binding protein (CREB)-binding protein; Dvl: Disheveled; LEF: Lymphocyte enhancer factor; P: Phosphorylation; PYG: Pygopus; Ub: Ubiquitin.

respects and they possess the capacity to generate progeny cells and self-renewal abilities, resulting in relapse and chemo-resistance [36,37]. Wnt/β-catenin signaling is activated in CSCs as well as in normal stem cells. The Wnt/β-catenin signaling pathway plays a crucial role in the maintenance of CSCs in many cancers, including colon, skin and breast cancer and leukemia [38-42]. Vermeulen *et al.* demonstrated the heterogeneity of Wnt signaling activity levels in a colon CSC population using the TCF/LEF reporter driving the expression of GFP [39]. These authors revealed that cells with high Wnt signaling activity possess clonogenicity. Moreover, colon cancer cells in which Wnt signaling is activated at high levels were found to be located close to stromal myofibroblasts. These observations suggest that the microenvironment regulates Wnt signaling activity in CSCs.

Several studies suggest that the epithelial-to-mesenchymal transition (EMT) is an important process in tumor progression [43,44]. EMT is the process by which epitherial cells lose their epithelial characteristics and acquire mesenchymal properties. EMT allows cancer cells to detach from the tumor mass and disseminate to other tissues [43,45]. Recent reports indicate that the accumulation of nuclear β-catenin is required for EMT, although it is not sufficient to induce EMT [46,47].

Table 1. Dysregulation of Wnt/β-catenin signaling pathway in cancer.

Gene	Alteration	Type of cancer	Ref.
Wnt ligands	Increased expression	Colon cancer	[95,96]
		Breast cancer	[62]
		Head/neck cancer	[97]
		Lung cancer (NSCLC)	[61]
		Gastric cancer	[98]
		Melanoma	[99]
		Prostate cancer	[100]
Frizzled receptors	Increased expression	Colon cancer	[95,101]
		Gastric cancer	[102]
		Synovial sarcoma	[103]
SFRP	CpG methylation and reduced expression	Colon cancer	[58,104,105]
		Breast cancer	[106,107]
		Gastric cancer	[102,108,109]
		Lung cancer	[110,111]
		Leukemia	[112]
		Bladder cancer	[113]
DKK	CpG methylation and reduced expression	Colon cancer	[56,114]
		Gastric cancer	[115]
		Breast cancer	[106]
WIF	CpG methylation and reduced expression	Breast cancer	[116,117]
		Bladder cancer	[117]
		Proatate cancer	[117]
		Lung cancer	[117,118]
APC	Inactivating mutation	Colon cancer	[119-121]
		Barret's esophagus	[122]
β-catenin	Activating mutation	Colon cancer	[123,124]
		Gastric cancer	[125,126]
		Hepatocellular cancer	[127,128]
		Hepatoblastoma	[128]
		Ovarian cancer	[129]
		Wilm's tumor	[130]
Axin	Inactivating mutation	Hepatocellular cancer	[128]
		Colon cancer	[131]

APC: Adenomatous polyposis coli; Dkk: Dickkopf; SFRP: Soluble frizzled-related proteins; WIF-1: Wnt inhibitory factor-1.

4. Wnt/β-catenin signaling pathway inhibitors for cancer therapy

Strategies to inhibit the Wnt/β-catenin signaling pathway have been eagerly developed for the treatment of cancers. Wnt/β-catenin signaling pathway inhibitors are classified roughly into two groups: biologic inhibitors and small-molecule compounds. Biologic inhibitors include monoclonal antibodies and recombinant nucleic acids (siRNA). Small-molecule compounds fall into two categories: conventional agents and novel agents. Compounds with inhibitory effects on the Wnt/β-catenin signaling pathway are contained in certain existing agents, such as NSAIDs and imatinib. The identification of novel compounds is done using various screening methods.

4.1 NSAIDs

NSAIDs are primarily used to treat inflammation, pain and fever. Epidemiological and clinical studies have shown that NSAIDs possess the ability to lower the risk of developing various type of cancers [48,49]. NSAIDs were also found to prevent the development of polyps and reduce tumor tissue in FAP patients [50]. Because COX-2 is highly expressed in various cancers [51], and it is also correlated with cancer progression, the effect of NSAIDs in preventing cancer progression is thought to be mediated by a direct inhibition of COX2. In addition, several studies indicate that NSAIDs inhibit Wnt/β-catenin signaling, which results in the prevention of cancer progression [12-14]. These results demonstrate a direct correlation between COX-2 and the Wnt/β-catenin signaling pathway.

Castellone *et al.* demonstrated the connection between COX-2 and Wnt signaling by showing that COX-2-derived prostaglandin E2 (PGE2) decreased phosphorylated β-catenin and increased nuclear β-catenin in colon cancer cell lines [52]. PGE2 released GSK3β from the destruction complex via the EP2 receptor, concurrently with the phosphorylation and inactivation of GSK3β through the PI3K/Akt signaling pathway, which was stimulated by PGE2.

However, some NSAIDs that do not possess COX2 inhibitory activity retain potent inhibitory effects on the Wnt/β-catenin signaling pathway in cancer cells, implying that the inhibition of this signaling pathway by NSAIDs results not only from COX2 inhibition but can also be mediated by other mechanisms [53,54]. Tuynman *et al.* described a mechanism of inhibition of Wnt/β-catenin signaling by NSAIDs [55]. Analysis of the activity of cellular kinases revealed that celecoxib impairs the auto-phosphorylation of the tyrosine kinase receptor c-Met, which results in the inhibition of Wnt/β-catenin signaling. The phosphorylation of c-Met leads to the activation of PI3K signaling and subsequent inactivation of GSK-3β. Consequently, dephosphorylation of c-Met by celecoxib induces a decrease of GSK-3β activity and the subsequent degradation of β-catenin.

4.2 Restoration of SFRP, WIF, and Dkk function

The decreased expression of secreted antagonists such as SFRP, WIF-1 and Dkk is observed in some cancers. SFRP and WIF-1 inhibit binding of the Wnt ligand and Fz receptor by binding directly to the Wnt ligand, while Dkk inhibits the Wnt signaling pathway by binding to LRP5/6. In some cancer cells, methylation of the promoter CpG island leads to a decrease in the expression of these Wnt antagonists. Efficient gene transfer of these antagonists has been evaluated as a therapeutic approach. Several reports showed that overexpression of Dkks inhibits cell growth in colon and lung cancer cells [56,57]. Moreover, the restoration of the function of SFRPs attenuated Wnt/β-catenin signaling in colon cancer cells, even in the presence of downstream mutations [58]. Similarly, the restoration of WIF-1 function attenuated Wnt signaling and induced apoptosis in colon cancer cells

harboring downstream mutations [59]. These reports indicated that restoration of the function of secreted antagonists might be a potent therapeutic approach to the treatment of certain tumors, despite the presence of downstream mutations.

4.3 Monoclonal antibodies

With regard to biological safety and specificity of treatment, antibody-based therapies provide an excellent therapeutic approach. Because small-molecule compounds can diffuse into the cells, they can affect intracellular targets. On the other hand, antibodies cannot cross the cell membrane and therefore do not target intracellular proteins, thus providing a safer treatment modality. Certain autocrine Wnt ligands contribute to cell proliferation and survival in various tumors [60,61]. Antibody-based therapies targeting Wnt ligand–Fz receptor binding at the cell surface can be effective in the treatment of certain cancers.

Upregulation of the Wnt-1 ligand (WNT-1) has been reported in various human cancers [9,62]. The activation of the Wnt/β-catenin signaling pathway by WNT-1 was found to correlate with tumor proliferation, and WNT-1 was therefore used as a direct target of treatment, as demonstrated by blocking WNT-1 signaling using an anti-WNT-1 monoclonal antibody, which induced apoptosis in WNT-1-overexpressing tumor cells, and suppressed tumor growth in mice [7-9]. The advantage of this treatment approach is that the anti-WNT-1 antibody has minimal effects in cells that do not express WNT-1 or express it at low levels, and it is therefore effective only in WNT-1-overexpressing cells, showing the specificity and selectivity of this treatment strategy.

4.4 siRNA

siRNA is an attractive therapeutic approach based on the induction of sequence-specific gene silencing [63]. In colon cancer cell lines, β-catenin siRNA reduced β-catenin expression and β-catenin/TCF-mediated transcription. In addition, β-catenin siRNA inhibited tumor growth in a SW480 xenograft model [11]. Moreover, work from our group demonstrated that β-catenin siRNA inhibited the growth of multiple myeloma tumors in a xenograft model [10]. Multiple myeloma (MM) is a human hematological malignancy characterized by the clonal expansion of plasma cells in bone marrow. β-catenin is overexpressed in MM cells compared with normal plasma cells, although the mechanisms underlying β-catenin overexpression are not clear. A recent study reported that β-catenin overexpression is caused by epigenetic dysregulation of the Wnt/β-catenin signaling pathway; this study also found that Wnt antagonists (WIF1, Dkk3, APC, SFRP1, SFRP2, SFRP4 and SFRP5) are methylated in MM cell lines and primary MM cells [64]. This dysfunction of Wnt antagonists induced the activation of the Wnt/β-catenin signaling pathway. siRNA-mediated blockade of Wnt/β-catenin signaling resulted in the inhibition of the progression of MM [10]. Because siRNAs induce gene-specific silencing, this treatment does not affect other signaling pathway or cause non-specific effects. β-catenin siRNA specifically inhibits β-catenin expression and therefore only suppresses activities downstream of β-catenin. However, problems associated with this treatment approach that have not yet been resolved include the inability of intravenously-administered siRNA to reach target tissues because siRNA is unstable in the bloodstream. If the stability of siRNA in the blood can be improved and a better drug delivery system can be designed, siRNA-based therapeutic approaches could become effective in the treatment of cancers.

β-catenin is a component of cadherin junctions and acts as a bridge connecting E-cadherin to actin filaments. The downregulation of β-catenin should therefore impair E-cadherin-dependent cell–cell adhesion. siRNAs should be designed to specifically target tumor cells to minimize the damage to normal adherent cells. This could be achieved through the conjugation of siRNA to an antibody against a tumor-expressed antigen [65].

4.5 Imatinib

The function of β-catenin is regulated by phosphorylation of different serine and threonine residues, namely S31, S33, T41 and S45, which are associated with its degradation. The phosphorylation of tyrosine residues plays a different role from serine/threonine phosphorylation and is important in determining the localization of β-catenin at the cell membrane, where it co-localizes with E-cadherin and interacts with β-catenin. Tyrosine 654 of β-catenin plays a role in the binding to E-cadherin and the phosphorylation of tyrosine 654 decreases the binding. Similarly tyrosine 142 is necessary for the interaction of β-catenin with α-catenin and the phosphorylation of tyrosine 142 decreases this interaction. These tyrosine residues are phosphorylated by specific tyrosine kinases: tyrosine 654 by EGFR and Src [66-68], and tyrosine 142 by Fyn, Fer and Met [66,69]. The phosphorylation of these tyrosine residues impairs E-cadherin–mediated cell adhesion and induces the activation of Wnt signaling by increasing cytoplasmic β-catenin.

Imatinib is a tyrosine kinase inhibitor used for the treatment of chronic myeloid leukemia (CML) [70,71]. Imatinib has been shown to inhibit the Wnt/β-catenin signaling pathway in colon cancer and thyroid cancer cell lines [15,16] through a mechanism involving the indirect inhibition of the tyrosine-phosphorylation of β-catenin, which caused a decrease in the accumulation of β-catenin in the nucleus.

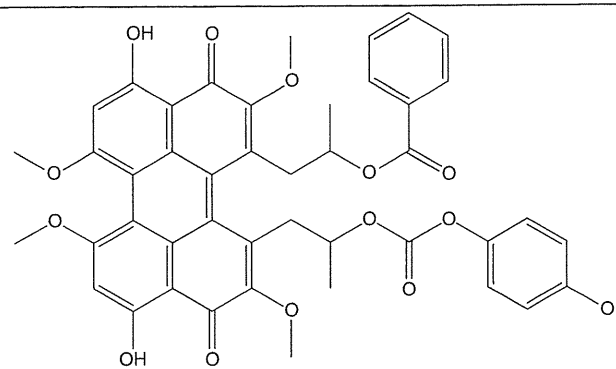
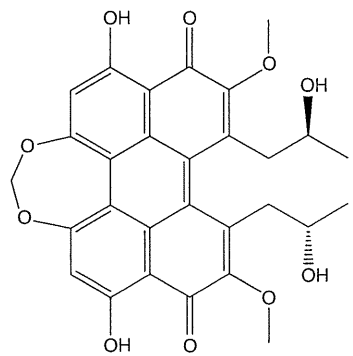
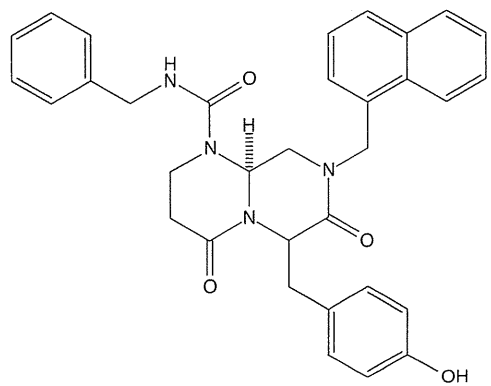
4.6 Small-molecule compounds

Small-molecule compounds have been developed extensively as therapeutic agents (Table 2) mainly due to their ability to target intracellular proteins. The screening of small-molecule compounds is crucial to their development. In the following sections, the small-molecule inhibitors that have been identified and the screening methods used are described in detail.

4.6.1 The screening of protein–protein interactions

The identification of small-molecule inhibitors based on the interaction between the components of a signaling pathway is an effective method in Wnt/β-catenin signaling due to the fact that this pathway is well characterized.

Table 2. Small-molecule inhibitors targeting the Wnt/β-catenin signaling pathway.

Name	Structure	Action	Ref.
PKF115 – 584		Prevention of β-catenin–TCF interaction	[74-77]
CGP049090		Prevention of β-catenin–TCF interaction	[74,76,77]
ICG-001		Prevention of β-catenin–CBP interaction	[79]

CBP: cAMP response element binding protein (CREB)-binding protein; Siah: Seven in absentia homolog; TCF: T-cell factor.